

ZINC STATUS RESPONSE TO FOLIC ACID SUPPLEMENTATION  
AND THE EFFECT OF LEVEL OF ZINC INTAKE ON  
FOLATE UTILIZATION IN HUMAN SUBJECTS

By

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I dedicate this dissertation to all of the people who were instrumental in helping me achieve this goal and to my steadfast, loyal and loving companions, Chelsea and Curly.

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## LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Meaning</u>
AAS	atomic absorption spectrophotometry
BSA	bovine serum albumin
C	Centigrade
$^{14}\text{C}$	carbon 14
cm	centimeter
$[\text{}^{14}\text{C}]\text{-PteGlu}_7$	carbon 14-labeled pteroylglutamylhexaglutamate
d	day
DPBS	Dulbecco's phosphate-buffered saline
$\text{D}_2\text{FA}$	deuterium-labeled folic acid
dL	deciliter
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	Fahrenheit
FA	folic acid
FBP	folate-binding protein
FIGLU	formimino-glutamic acid
FDA	Food and Drug Administration
g	gram
<i>g</i>	gravity

GCMS	gas chromatography-mass spectrometry
h	hour
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
[ <sup>3</sup> H]PteGlu	tritiated pteroylmonoglutamic acid
HPV-16	human papillomavirus 16
IU	International Units
kcal	kilocalories
kg	kilogram
L	liter
M	molar
mg	milligram
mL	milliliter
mol	mole
mmol	millimole
MRC	Medical Research Council
MT	metallothionein
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
NaN <sub>3</sub>	sodium azide
ng	nanogram
nm	nanometer
oz	ounce
P	probability
pABG	para-aminobenzoyl glutamate

PBS	phosphate-buffered saline
ppm	parts per million
RDA	Recommended Dietary Allowances
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
THF	tetrahydrofolate
$\mu\text{Ci}$	microcurie
$\mu\text{g}$	microgram
$\mu\text{L}$	microliter
$\mu\text{M}$	micromolar
$\mu\text{mol}$	micromole
v/v	volume/volume
y	year
Zn	zinc

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Changes in zinc status in response to folic acid supplementation and the effect of level of zinc intake on folate utilization were evaluated in human subjects. Twelve healthy men were randomly assigned to consume a zinc-restricted diet (3.5 mg zinc/d) or an identical diet supplemented with 11 mg of zinc as zinc sulfate (14.5 mg zinc/d total) for 25 days. Half of the subjects in each group received 800  $\mu$ g/d deuterium-labeled folic acid. The remaining subjects received a placebo. After an 80-day washout period, the study was repeated with the folic acid/placebo treatments reversed. Fasting blood samples and 24-hour urine collections were obtained at corresponding time points during both study periods.

Plasma zinc, erythrocyte zinc, urinary zinc, serum alkaline phosphatase and erythrocyte metallothionein

concentrations were measured to determine zinc status. No differences in mean values within or between the zinc diet groups were detected ( $P \geq 0.05$ ) for these response variables. A significant ( $P < 0.001$ ) time by supplement by diet interaction was detected for erythrocyte metallothionein; however, significant differences in the change in erythrocyte metallothionein concentrations due to the supplemental effect were not detected ( $P \geq 0.05$ ) within the zinc diet groups. No significant differences due to the effect of the supplement were detected ( $P \geq 0.05$ ) within or between the zinc diet groups for the remaining response variables. These data suggest that short-term supplementation with 800  $\mu\text{g}$  folic acid/d does not adversely affect zinc status in healthy men.

To determine the effect of zinc intake on folate utilization, total urinary, serum and erythrocyte folate concentrations, and urinary excretion of deuterium-labeled and unlabeled folate were determined. Differences in mean serum, erythrocyte, total urinary or urinary deuterium-labeled folate concentrations between subjects fed 3.5 or 14.5 mg zinc/d were not detected ( $P \geq 0.05$ ). Similarly, significant differences in the percent of total folate ingested or the percent of total urinary folate excreted as deuterium-labeled folate were not detected ( $P \geq 0.05$ ) between the groups. These data suggest that utilization of supplemental folic acid in healthy men is not influenced by the level of zinc intake.



## CHAPTER 1 INTRODUCTION

The crucial roles of folate and zinc in protein and nucleic acid metabolism and genetic expression have contributed to the intense research interest centered around these two nutrients. Changes in intake, bioavailability and/or metabolism of either or both of these nutrients can have deleterious effects in humans and animals. Understanding the specific mechanisms of action and relationships between zinc and folate is essential to improving our ability to make efficacious nutritional recommendations and policies that will enhance the health and well-being of the human race.

Zinc is an integral constituent of at least 60 different enzymes (Cousins and Hempe, 1990) including deoxyribonucleic acid (DNA) transferase, ribonucleic acid (RNA) polymerases, alkaline phosphatase, gamma aminolevulinic acid dehydratase, carbonic anhydrase, carboxypeptidase, alcohol dehydrogenase and glutamic, lactic and malic acid dehydrogenases (Vallee and Galles, 1984). Of particular interest with regard to studies investigating the relationship between zinc and folate nutrition was the discovery (Silink et al., 1975) that bovine hepatic folate conjugase is a zinc metalloenzyme. The fact that zinc is a component of so many metalloenzymes explains

the multiplicity of physiologic functions attributed to this nutrient. Examples of these functions include development and maintenance of the body's immune system, prevention of lipid peroxidation, metabolism of energy containing nutrients, hormonal interactions, bone formation and the replication and differentiation of cells (Cousins, 1985; Cunnane, 1988; Hambidge et al., 1986).

"Folate" is the generic term used in reference to the many different naturally occurring forms of pteroylmonoglutamic acid and pteroylpolyglutamic acid. These compounds have nutritional properties and chemical structures similar to their parent compound, folic acid. Folate is a coenzyme for many one carbon reactions, and like zinc, is required for cell replication. Specifically, folate coenzymes are essential for the synthesis of the pyrimidine, thymidylate, which is required for DNA synthesis. Other metabolic processes requiring folate coenzymes include the interconversion of serine and glycine, methionine synthesis, histidine degradation, methylation of biogenic amines, generation of formate and purine biosynthesis (Brody, 1991). Recent studies suggest that supplemental folic acid may be important in reducing the incidence of neural tube defects (Bower and Stanley, 1989; Czeizel and Dudás, 1992; Medical Research Council Vitamin Study Research Group, 1991; Milunsky et al., 1989; Mulinare et al., 1988; Smithells et al., 1981); modulating cancer risk in a variety of tissues including the

lung (Heimbürger et al., 1987; 1988), cervix (Butterworth et al., 1982; 1992a; 1992b), esophagus (Jaskiewicz et al., 1988) and colon (Lashner et al., 1989); and reducing elevated homocysteine levels, an emerging independent risk factor for coronary heart disease (Kang et al., 1986; 1987).

Despite the potential benefits associated with folic acid supplementation, and the fact that it is widely believed to be nontoxic to humans under normal circumstances (DiPalma and Ritchie, 1977), concern has been expressed regarding the relative safety of ingesting additional amounts of this nutrient. The major safety issues as outlined by Butterworth and Tamura (1989) focus on: the potential harm to users of anticonvulsant medications; interference with the diagnosis of vitamin B<sub>12</sub> deficiency; the possibility of other unexpected adverse health effects; and interference with zinc absorption or metabolism. For the most part, all but the latter safety issue have been adequately addressed.

The question of whether supplemental folic acid disturbs zinc absorption or metabolism, and thus zinc status, has been examined by numerous researchers (Butterworth et al., 1988; Fuller et al., 1987; Ghishan et al., 1986; Keating et al., 1987; Krebs et al., 1988; Milne, 1989; Milne et al., 1984; Mukherjee et al., 1984; Simmer et al., 1987; Tamura et al., 1992; Wilson et al., 1983). The results from these studies have been equivocal. Of these studies, dietary intake was controlled in only one research design (Milne et al., 1984);

consequently, the results and conclusions of these studies may vary depending on the adequacy and comparability of intake among subjects. Discrepant results may also be attributed to the diversity of research designs and protocols used, as well as the lack of a satisfactory index of zinc nutriture.

The present study was designed to overcome some of the limitations of previous investigations by evaluating the response of male human subjects to 0  $\mu\text{g/d}$  and 800  $\mu\text{g/d}$  of stable-isotopically labeled (deuterium) folic acid under controlled dietary conditions. The objective was to determine if supplemental folic acid affected zinc status in subjects consuming zinc-adequate (i.e. 14.5 mg/d) or zinc-restricted (i.e. 3.5 mg/d) diets. Additionally, the use of deuterium-labeled folic acid provided the opportunity to study folate utilization under conditions of marginal and adequate zinc intakes. This is the first time that folate utilization using a stable isotope has been studied under controlled dietary conditions. Another significant aspect of this study was the determination of erythrocyte metallothionein concentrations in addition to traditional measures of zinc status. Unlike other indices of zinc status, the concentration of erythrocyte metallothionein has been shown (Grider et al., 1990) to respond quickly to acute dietary zinc deficiency and to supplementation and may therefore be a more reliable and sensitive indicator of zinc status.

## CHAPTER 2 REVIEW OF THE LITERATURE

### Folate

#### Chemistry

Folic acid, or pteroylglutamic acid (2-amino-4-hydroxy-6-methyleneaminobenzoyl-L-glutamic acid pteridine), consists of three distinct subunits: a pteridine moiety, para-aminobenzoic acid and glutamic acid (Figure 2-1). The pteridine moiety is linked by a methylene bridge to para-aminobenzoic acid, which is then joined by peptide linkage to glutamic acid. Although mammals can synthesize all the components of this vitamin, they are not capable of de novo biosynthesis because they lack the enzyme needed for coupling the pteridine molecule to para-aminobenzoic acid (Cooper, 1984). De novo synthesis of folates does occur in plants and bacteria.

Folic acid is yellow and has a molecular weight of 441.4. It is only slightly soluble in water in the acid form, but is quite soluble in the salt form (Brody, 1991). Folic acid occurs only rarely in nature, although it is the form most commonly found in vitamin supplements and is the parent compound of the naturally occurring folate vitamin forms.



Compounds having nutritional properties and chemical structures similar to those of folic acid have been assigned the generic descriptor "folate." Natural folates occur in the reduced 7,8-dihydro- and 5,6,7,8-tetrahydro- forms. Folates may also contain one-carbon substituent groups (Figure 2-1). These include: 5-methyl-, 10-formyl-, 5-formyl-, 5,10-methenyl-, 5,10-methylene-, and 5-formimino-tetrahydrofolate (Brody, 1991). Most of these naturally occurring folates can be degraded by heat, oxidation and/or ultraviolet light, although some tetrahydrofolate derivatives are more stable than others. For example, N<sup>5</sup>-methyl-tetrahydrofolate is relatively heat stable, but is destroyed by acid, as is N<sup>5,10</sup>-methylene- tetrahydrofolate (Krumdieck, 1990; O'Brion et al., 1975). For the most part, dietary folates occur in the form of pteroylpolyglutamates (Halsted, 1979) containing three to seven glutamic acid residues (Figure 2-1). Although the principal pteroylpolyglutamate in food is N<sup>5</sup>-methyl-tetrahydrofolate, over 150 different forms of folate have been reported to exist (Sauberlich, 1987).

### Metabolism

Absorption. The first stage of intestinal folate absorption involves the hydrolysis of pteroylpolyglutamates to pteroylmonoglutatmes (Butterworth et al., 1969). Hydrolysis is performed by the gamma glutamylcarboxypeptidases, commonly grouped together and referred to as "folate conjugase".

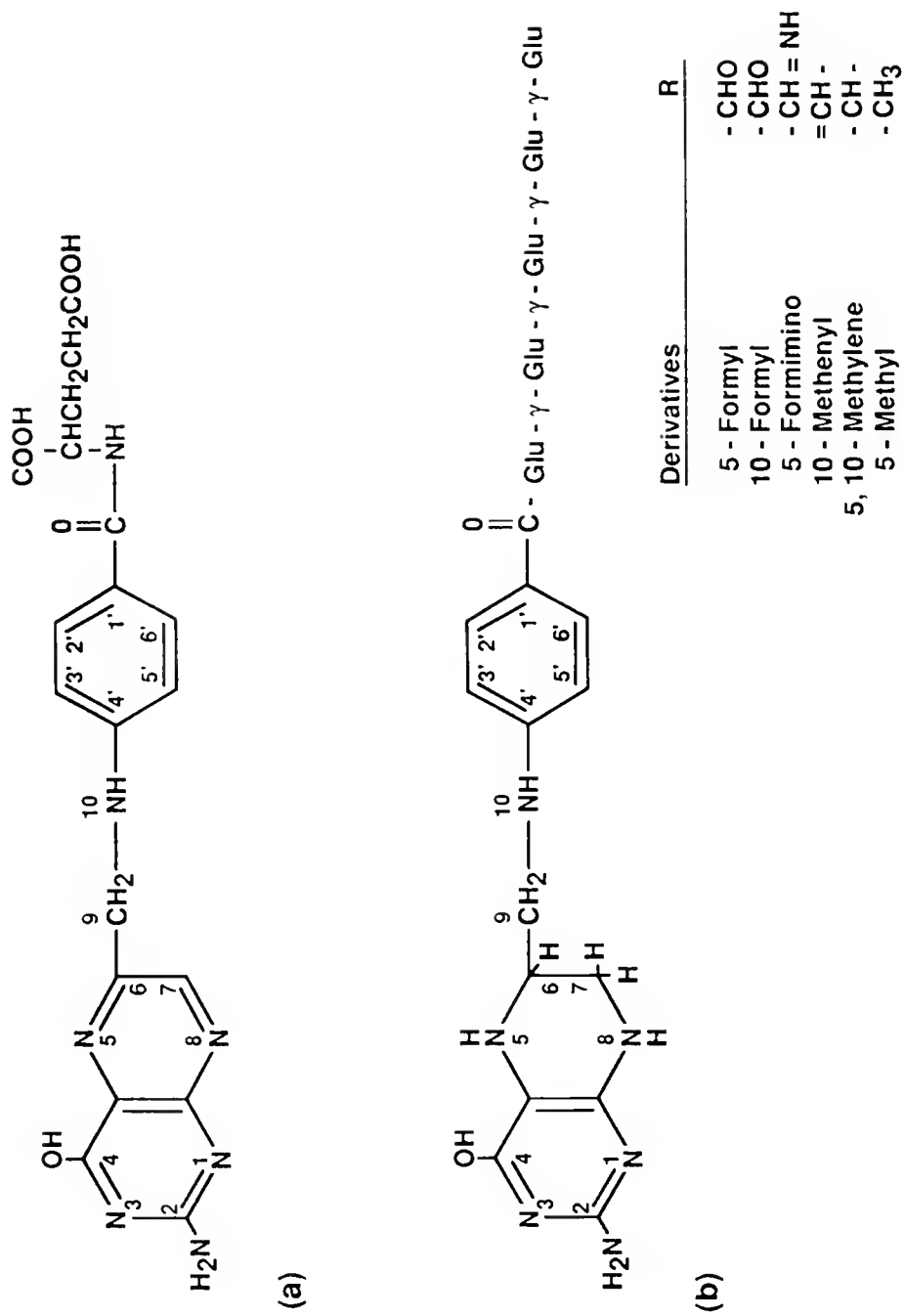


Figure 2-2. Folate-mediated one-carbon metabolism (Brody, 1991).



Folate conjugase successively cleaves the gamma glutamyl peptide bonds of pteroylpolyglutamates to the monoglutamate form. Reisenauer and Halsted (1987) have estimated that the activity of human jejunal brush border folate conjugase is sufficient enough to preclude this step from limiting the rate of absorption. However, research showing that the bioavailability of folate polyglutamates is considerably less than that of folate monoglutamates (Gregory et al., 1991) suggests that hydrolysis of dietary polyglutamyl folates is a rate-limiting step in absorption.

Two separate folate conjugases have been identified in the human jejunum (Reisenauer et al., 1977). One of these is soluble and located intracellularly, while the other is membrane-bound and concentrated in the brush border. These folate conjugases differ with respect to molecular weight, pH optima and inhibition characteristics.

Brush border folate conjugase is a zinc-dependent exopeptidase that sequentially cleaves polyglutamates to monoglutamates and has a pH optimum near neutrality (Chandler et al., 1986; Reisenauer et al., 1977). Chronic alcohol consumption (Naughton et al., 1989; Reisenauer et al., 1989), zinc deficiency (Tamura et al., 1978), mucosal damage (Halsted, et al., 1986) or exposure to naturally occurring inhibitors in food (Bhandari and Gregory, 1990) may exert a negative effect on brush border folate conjugase activity and subsequent folate absorption. Interestingly, brush border

folate conjugase activity does not appear to be affected by the aging process (Bailey et al., 1984a). In contrast to brush border folate conjugase, intracellular folate conjugase is found in the lysosomes of intestinal cells and functions as an endopeptidase with an acidic pH optimum (Wang et al., 1986). The role of intracellular folate conjugase is unknown; however, Wang et al. (1986) have proposed that its role in cellular folate metabolism is unrelated to the digestion of dietary folates.

Transport of folate across the brush border membrane is the second stage of folate absorption. This is a complex process that has not been entirely elucidated. Transport occurs mainly in the jejunum and is believed to involve a carrier system that is saturable, pH dependent (i.e. pH optimum of 6.0), energy dependent and sodium dependent (Rose et al., 1978; Said et al., 1987; Selhub et al., 1983). This carrier-mediated system is thought to include a folate-binding protein located in the jejunal brush border membrane. This folate-binding protein is either the transport protein or is an important component of the intestinal transport system (Reisenauer, 1980). Data from competitive inhibition studies (Said, et al., 1987; Selhub et al., 1984) support the conclusion that the transport system may be the same for all monoglutamate forms of folate. The carrier-mediated system becomes saturated at luminal concentrations of 10-20  $\mu$ M (Selhub et al., 1984). In addition to carrier-mediated

transport, nonsaturable absorption involving passive diffusion may also occur (Selhub et al., 1983). Absorption of folate by this process occurs linearly at much higher folate concentrations.

Decreased hydrolysis of folate polyglutamates and/or interference with the transport of folate monoglutamates across the intestinal brush border membrane can inhibit folate absorption. Studies concerning the impact of dietary components (i.e. conjugase inhibitors, dietary fiber, etc.); dietary composition; the actual forms of folate ingested; nutritional status; alcohol consumption; and the effect of nutrient interactions on the extent of folate absorption have been reviewed by Bailey (1988). The conclusions drawn from these studies are often conflicting, confirming the need for further research employing more consistent and appropriate experimental designs and methodologies. A case in point is the nutrient interaction that is hypothesized to occur between zinc and folic acid.

Nondietary factors that may interfere with folate absorption include altered gastrointestinal function and the use of medications. Damage to the intestinal epithelial cells, such as that which occurs in Crohn's disease (Hoffbrand et al., 1968), celiac disease (Halsted et al., 1977; 1978; Hoffbrand et al., 1970) and tropical sprue (Corcino et al., 1976; Halsted, 1980b), or changes in the intraluminal environment due to achlorhydria (Russell et al., 1986), may

adversely affect folate absorption. Medications that may interfere with folate absorption include sulfasalazine (Franklin and Rosenberg, 1973) and diphenylhydantoin (Gerson et al., 1972; Hoffbrand and Necheles, 1968; Rosenberg et al., 1968). Antacids and histamine receptor antagonists may also adversely affect folate absorption by increasing the intestinal pH to levels that exceed the optimum for folate conjugase activity and carrier-mediated transport (Russell et al., 1979). Folate analogs such as methotrexate, trimethoprim and pyrimethamine, which are used primarily as dihydrofolate reductase inhibitors, also suppress intestinal folate absorption (Selhub et al., 1983).

Transport. Upon entry into the intestinal mucosal cells, reduced dietary folates are converted, for the most part, to N<sup>5</sup>-methyl-tetrahydrofolate monoglutamate (Cooper, 1984; Pratt and Cooper, 1971). This is the predominant form in the portal circulation under normal physiologic conditions. Reduction and methylation of folic acid can also occur, although much of this form of the vitamin appears in the portal blood unchanged (Cooper, 1984).

Serum folate binding proteins are involved with the transport and distribution of folates to the liver and other tissues. Two types of serum folate-binding proteins have been identified (Wagner, 1985), one is specific with a high-affinity binding capacity; the other, which is thought to be albumin (Soliman and Olesen, 1976), is nonspecific, with a

low-affinity binding capacity. Approximately two-thirds of serum folate is protein-bound, with the majority bound to albumin.

A small amount (10 to 20%) of absorbed folates in reduced substituted form is taken up by the liver on the first pass, while the majority is distributed to other tissues (Steinberg, 1984). Since the liver is capable of secreting N<sup>5</sup>-methyl-tetrahydrofolate monoglutamate into the bile, the potential exists for continual enterohepatic circulation of folate (Steinberg, 1984). This idea is not supported, however, by the results of an isotopic labeling study (Krumdieck et al., 1978) that examined the routes of folate excretion following administration of [<sup>14</sup>C]folic acid to a human subject. The results of this study suggested that a significant portion of biliary folate was not reabsorbed.

Contrary to the fate of reduced substituted forms of folate entering the portal hepatic vein, folic acid entering this vessel is taken up almost exclusively by the liver. Some of this folic acid is used for polyglutamate synthesis, and the rest is converted to N<sup>5</sup>-methyl-tetrahydrofolate and secreted into the bile (Lavoie and Cooper, 1974). When a large oral dose of folic acid is administered, most of it is recovered in the urine in its original form (Brody, 1991).

Tissue deposition and storage. Transport of folate across cellular membranes is an energy requiring process, where anion gradients may serve as the energy source

(Yang et al., 1984). The N<sup>5</sup>-methyl-tetrahydrofolate transported across cell membranes must be converted to the polyglutamate form to assume its role as a functional coenzyme. Polyglutamates cannot cross biological membranes, so polyglutamation also serves to trap folates inside cells at concentrations that are one to two orders of magnitude greater than those of the extracellular fluid. Conversion to the polyglutamate form requires methionine synthetase, a vitamin B<sub>12</sub>-dependent enzyme, and pteroylpolyglutamate synthetase. Methionine synthetase is responsible for removing the methyl group, and pteroylpolyglutamate synthetase is responsible for the addition of glutamyl residues. The resulting tetrahydrofolate polyglutamates usually contain between four to seven glutamyl residues.

The total body folate content is in the range of 5 to 10 mg. The liver is considered to be the primary storage organ containing about 50% of the total body folate (Herbert and Colman, 1988). A variety of folate derivatives with various chain lengths is present in the liver and other tissues, with one particular species usually dominating (Shane, 1990). Alterations in the distribution of folate polyglutamates have been observed under certain physiological and nutritional conditions. For example, longer chain length folates tend to accumulate under circumstances whereby cellular folate concentrations are diminished (i.e. folate deficiency; methionine deficiency; etc.) (Cook et al., 1987). The

significance of this particular change has not been determined, although it has been reasoned that extending the chain length does not affect the short-term regulation of one-carbon metabolism since this change occurs very slowly (Shane, 1990).

Utilization of endogenous folate pools may provide a mechanism for maintaining normal folate supplies to critical tissues during acute or chronic folate deprivation. A small pool of intracellular folate monoglutamates is thought to be available for this purpose. As deprivation continues, the liver and other "storage tissues" (i.e. the kidney) may generate folate monoglutamates through the action of folylpolyglutamyl hydrolase on folate polyglutamates. Additionally, the amount of folate presented to the liver is thought to decrease, thereby reducing hepatic monoglutamate uptake and new polyglutamate synthesis. Thus, over time, hepatic folate stores decline.

Folate derived from dying cells may be another potential source of this nutrient during periods of prolonged deprivation (Steinberg, 1984). For example, erythrocytes could make an important contribution to folate homeostasis because of their high folate content and limited life span. A study (Hillman et al., 1982) of heat-treated, senescent erythrocytes has shown that labeled folate can be recovered in the liver and bile. Therefore, folate salvaged from expired

erythrocytes may be redistributed through the enterohepatic circulation.

Catabolism and excretion. Urinary excretion of intact folates in well-nourished humans consuming a nutritionally adequate diet is approximately 5-40  $\mu\text{g}$  per day (Herbert, 1987). The folate compounds identified in the urine include  $\text{N}^5$ -methyl-tetrahydrofolate,  $\text{N}^{10}$ -formyl-tetrahydrofolate,  $\text{N}^{5,10}$ -methenyl-tetrahydrofolate and  $\text{N}^5$ -formyl-tetrahydrofolate (Chanarin, 1979). Excretion of intact folates is limited due to renal tubular reabsorption of this nutrient and the degree of catabolism that occurs in vivo. Products of folate catabolism occurring in the urine include pteridines, para-acetamidobenzoyl glutamate and para-acetamidobenzoate (Anon., *Nutrition Reviews*, 1990; Chanarin, 1979). The latter two catabolites appear to be the major excretory products, suggesting that the principal route of catabolism occurs by cleavage of the  $\text{C}^9$ - $\text{N}^{10}$  bond. The cleavage mechanism has been the subject of much controversy. Current in vitro studies suggest the existence of more than one mechanism for folate cleavage (Anon., *Nutrition Reviews*, 1990).

Folate can also be excreted in the feces. The amount excreted in the feces has been reported, in some cases, to be higher than the estimated dietary intake, presumably because of folate synthesis by colonic bacteria (Brody, 1991). Consequently, fecal folate excretion is not considered a reliable index of folate metabolism.



### Biochemical Functions

Reduction of the pteridine ring to the tetrahydro-form, elongation of the glutamyl side chain and acquisition of one-carbon units at the N<sup>5</sup> and/or N<sup>10</sup> positions of the pteridine ring system must occur in order to produce the intracellular, metabolically active forms of folate. Pteridine ring reduction is accomplished by the cytosolic enzyme, 7,8-dihydrofolate reductase. Folic acid and dihydrofolic acid can serve as substrates for this enzyme. Reduction to the tetrahydrofolate form, and demethylation, in the case of N<sup>5</sup>-methyl-tetrahydrofolate, must occur before elongation of the side-chain or acquisition of one-carbon substituent groups can proceed. Side-chain elongation is achieved by the action of folate polyglutamate synthetase. This is an adenosine triphosphate (ATP)-dependent enzyme which joins glutamyl residues to the vitamin by peptide bonds in an oligo-gamma-glutamyl linkage. Once these steps have been accomplished, one-carbon units at the oxidation level of formate, formaldehyde or methanol can be added. The major contributor of one-carbon groups is serine, although formimino-glutamate (FIGLU), formylglutamate and formate may also serve as the single-carbon source. The resulting tetrahydrofolate derivatives (N<sup>5</sup>-methyl-, N<sup>5</sup>-formyl-, N<sup>10</sup>-formyl-, N<sup>5,10</sup>-methylene-, N<sup>5,10</sup>-methenyl- and N<sup>5</sup>-formimino-) are used as coenzymes and serve as donors and acceptors of one-carbon units in a host of reactions involved in amino acid and

nucleotide metabolism. Examples of specific biochemical functions of folate include: purine and pyrimidine biosynthesis; the generation and utilization of formate; and amino acid interconversions including the catabolism of histidine to glutamic acid, the interconversion of serine and glycine and the conversion of homocysteine to methionine. These reactions are collectively referred to as one-carbon metabolism (Brody, 1991; Krumdieck, 1990).

The cyclical nature of intracellular folate metabolism is exemplified by tracing the potential fates of tetrahydrofolate polyglutamates (see Figure 2-2). In mammalian tissues, it appears that the major cycle of one-carbon metabolism involves the conversion of tetrahydrofolate polyglutamate to  $N^{5,10}$ -methylene-tetrahydrofolate by serine hydroxymethyltransferase. This one-carbon derivative is a key intermediate that can be: oxidized to  $N^{5,10}$ -methenyl-tetrahydrofolate (by  $N^{5,10}$ -methylene-tetrahydrofolate dehydrogenase) for use in de novo synthesis of purines; used for the synthesis of thymidylate (by thymidylate synthetase); or reduced to  $N^5$ -methyl-tetrahydrofolate (by  $N^{5,10}$ -methylene-tetrahydrofolate reductase) for use in the biosynthesis of methionine. The latter two reactions are irreversible reactions that compete for the  $N^{5,10}$ -methylene-tetrahydrofolate intermediate. Regardless of the metabolic path into which the  $N^{5,10}$ -methylene-tetrahydrofolate is directed, its eventual fate (under normal physiologic conditions) is the loss of its one-

carbon substituent group, and its return to either dihydro- or tetrahydro-folate polyglutamate (Brody, 1991; Krumdieck, 1990).

Regulation of one-carbon transfer reactions is highly complex. Changes in the concentration of substrates, products and cofactors serve as a means for quickly activating or inhibiting certain folate-requiring reactions. An example of this complex regulatory process is one that has been given much attention and involves the fate of  $N^{5,10}$ -methylene-tetrahydrofolate. As noted above and depicted in Figure 2-2, this intermediate can be used to synthesize several different products. Methylene-tetrahydrofolate reductase, the enzyme catalyzing the conversion of  $N^{5,10}$ -methylene-tetrahydrofolate to  $N^5$ -methyl-tetrahydrofolate, is very highly regulated. When insufficient amounts of methionine are available, inhibition of the reductase enzyme is relaxed allowing for increased production of methionine from homocysteine. The methionine produced by this reaction is converted to S-adenosyl methionine, which then serves as a methyl donor to form various methylated products. However, when S-adenosyl methionine accumulates, methylene-tetrahydrofolate reductase activity is curtailed through feedback inhibition. Methylene-tetrahydrofolate reductase is also inhibited by the accumulation of dihydrofolate polyglutamates, a situation that occurs when thymidylate synthesis increases. Inhibition of this enzyme under these circumstances allows for continued

commitment of folate to nucleotide biosynthesis (Brody, 1991; Krumdieck, 1990).

In addition to the regulatory influences posed by changes in the concentration of substrates, products and cofactors, evidence for a second type of regulation has been reviewed by Krumdieck (1990). This newly proposed regulatory process is thought to be a slow-response mechanism based on covalent modification of the polyglutamyl chain length. The idea that a second form of regulation existed was generated from *in vivo* studies demonstrating that the glutamyl chain length of cellular folates varies in response to physiological or pathological stimuli (i.e. developmental age, tissue regeneration, infection, starvation, alcohol ingestion and methionine-choline deficiency) that alter the steady-state equilibrium of one-carbon metabolism. Additionally, the finding that chain-length distribution of folate polyglutamates differs from organ to organ in the same species adds credibility to this concept because it is unlikely that the requirements for one-carbon transfer reactions are the same for all organs. Since changes in polyglutamate chain length are slow to develop and only respond to persistent stimuli, it is thought that the purpose of this regulatory mechanism is to correct prolonged deviations rather than brief fluctuations that may occur in an otherwise steady-state (Krumdieck, 1990).

Table 2-1. Comparison of 1980 and 1989 Recommended Dietary Allowances (RDA) for folate.

Category/age	<u>1980 RDA*</u>	<u>1989 RDA**</u>
	$\mu\text{g/d}$	$\mu\text{g/d}$
-----		
Infants		
0.0-0.5	30	25
0.5-1.0	45	35
-----		
Children		
1-3	100	50
4-6	200	75
7-10	300	100
-----		
Men		
11-14	400	150
15-18	400	200
19-24	400	200
25-50	400	200
51+	400	200
-----		
Women		
11-14	400	150
15-18	400	180
19-24	400	180
25-50	400	180
51+	400	180
-----		
Pregnant women	800	400
-----		
Lactating women		
First 6 months	500	280
Second 6 months	500	260
-----		
*Data from Food and Nutrition Board, 1980.		
**Data from Food and Nutrition Board, 1989.		

### Recommended Dietary Allowances for Folate

The 1989 Recommended Dietary Allowances (RDA) for folate (Food and Nutrition Board, 1989a) represent a significant

reduction from the previously recommended amounts (Food and Nutrition Board, 1980) for all age, sex and special population categories (see Table 2-1). The rationale for the reduction of the RDA for folate was based on two types of data: the quantity of folate required to invoke established physiologic responses or replace daily losses, after adjusting for bioavailability and individual variability; and estimates of dietary folate consumption related to the prevalence of deficiency in population groups (Bailey, 1990a; Bailey, 1992). Although some researchers (Herbert, 1987; Reisenauer and Halsted, 1987) support the view that the existing data are sufficient to warrant a reduction in the recommended level of folate intake, others (Bailey, 1992; Sauberlich et al., 1987) cast doubt on the appropriateness of the new RDA. In a critique of the research used to establish the 1989 RDA, Bailey (1992) submits that this new RDA may be insufficient to provide an adequate margin of safety for specific populations. The rationale for her conclusion is based on the following facts: 1) in some cases, the population surveys used to make conclusions about folate requirements were not specifically designed to assess folate status; 2) the studies used to support the 1989 RDA were not always comparable in terms of the forms of folate used (i.e. synthetic folic acid versus dietary folate); 3) the correction factor used to account for folate bioavailability is only an estimate based on limited data from studies employing different experimental

methodologies; and 4) the food composition tables for folate are incomplete and may not accurately reflect the actual amount of folate ingested or available. This final problem is due to the fact that estimates of the folate content of foods vary due to the method of analysis; the type of food consumed; the method of food processing, preparation, storage and handling; and the effect of nutrient interactions.

#### Effects of High Doses of Folic Acid

Folate is considered nontoxic in small doses as well as in doses that exceed the RDA several hundredfold (Brody, 1991; Butterworth and Tamura, 1989; DiPalma and Ritchie, 1977; Herbert and Colman, 1988). The water soluble nature of folate, and the apparent requirement for attachment to saturable folate-binding proteins as a condition for storage, probably account for the relative nontoxicity of this vitamin. These features provide a mechanism for rapid excretion of folate when the serum- and tissue-binding capacity are exceeded (Herbert and Colman, 1988).

Adverse effects of supplemental folic acid were not noted in adult humans receiving 400 mg/d for 5 months, or after 10 mg/d for 5 years (Brody, 1991). Similarly, no adverse effects were reported in a group of well-nourished women ingesting an oral folic acid supplement (10 mg/d) for 4 months (Butterworth et al., 1982). Contrary to these reports, insomnia and irritability were noted in subjects consuming folic acid in

the amount of 15 mg/d (Hunter et al., 1970). This effect has not been confirmed in subsequent studies (Alhadeff et al., 1984).

Despite the apparent relative safety of supplemental doses of folic acid in normal adult subjects, large doses of this nutrient (5 mg/d) (Brody, 1991) can have deleterious effects when administered to individuals with undiagnosed and/or untreated pernicious anemia. Administration of folic acid supplements obscures the diagnosis of pernicious anemia by correcting the macrocytic anemia associated with this condition, but fails to alleviate the concurrent neurologic lesions. Consequently, neurologic damage progresses unchecked. Assessment of vitamin B<sub>12</sub> status prior to the initiation of folic acid supplementation can prevent this potentially harmful outcome.

Large doses of folic acid (100 or more times the RDA) may also be harmful to individuals with epilepsy who are receiving continuous phenytoin therapy. Supplementation at this level may precipitate convulsions (Herbert, 1987). Lower doses of folic acid (0.1 to 1 mg/d) have not been shown to impair seizure control (Roe, 1989).

It has been suggested that high intakes of folic acid may interfere with zinc absorption and/or metabolism (Ghishan et al., 1986; Milne, 1989; Milne et al., 1984; Mukherjee et al., 1984; Simmer, et al., 1987; Wilson et al., 1983). This topic



will be addressed in more detail in a subsequent section of this dissertation.

In contrast to some of the potentially harmful effects of supplemental folic acid, there is evidence to suggest that additional amounts of this vitamin may be beneficial in modulating cancer risk in a variety of tissues including cervical dysplasia (Butterworth et al., 1982; Butterworth et al., 1992a; Butterworth et al., 1992b), bronchial metaplasia (Heimbürger et al., 1987; Heimbürger et al., 1988), and neoplasms associated with the esophagus (Jaskiewicz et al., 1988) and colon (Lashner, et al., 1989). Supplemental folic acid has also been associated with a reduction in the risk of occurrence/recurrence of neural tube defects (Bower and Stanley, 1989; Czeizel and Dudás, 1992; Medical Research Council Vitamin Study Research Group, 1991; Milunsky et al., 1989; Mulinare et al., 1988; Smithells et al., 1981).

#### Sources/Distribution and Stability of Folate in Foods

Although there is a need for more complete data concerning the folate content and bioavailability of foods, a considerable amount of information concerning food sources of folate and the forms of the vitamin present in these foods has been generated. The most concentrated sources of folate include liver, citrus fruits, raw broccoli and dark green leafy vegetables such as raw spinach. Cooked greens, including spinach, turnip and mustard greens also contain

folate, but in lower amounts. The reduction in folate content due to cooking occurs primarily due to leaching of the vitamin into the cooking water, although thermal and oxidative destruction may also occur. Legumes are another highly concentrated source of folate; however, it must be recognized that they contain heat-activated conjugase inhibitors which may decrease the availability of folate polyglutamates. This is true for other folate containing foods such as cooked cabbage. Other good sources of folate include fortified breakfast cereals. Most fortified cereals supply at least 25% of the United States Recommended Daily Allowance for this nutrient. In addition to the more concentrated sources of dietary folate, it is important to consider the potential contribution that foods containing modest amounts of folate can have on total intake if these foods are consumed frequently and/or in large quantities. Examples of these include ground beef and whole-grain breads and cereals, as well as tea (Bailey, 1990a; Bailey, 1992).

Very little folic acid is naturally present in food, although this is the form used in food fortification because of its exceptional stability characteristics (Gregory, 1989). Folates in food occur almost exclusively in reduced polyglutamate forms, with the predominant forms being N<sup>5</sup>-methyl-tetrahydrofolate, N<sup>10</sup>-formyl-tetrahydrofolate and unsubstituted tetrahydrofolate polyglutamates of varying chain-lengths (Gregory, 1989). Reduced folates, with the

exception of N<sup>5</sup>-formyl- tetrahydrofolate, are potentially more labile and subject to oxidation under aerobic conditions, especially in the presence of heat, light and/or metal ions (Cooper et al., 1978; Gregory, 1989). The actual stability of folate in foods has been difficult to determine, with reported losses of folate activity being highly variable. This variability may be due to differences in oxygen exposure during cooking, the amount of cooking water present and/or intrinsic differences in ascorbic acid content of different foods (Gregory, 1989). When partial or full oxidation of tetrahydrofolate derivatives occurs, they may be further catabolized to yield compounds that are physiologically inactive with respect to human nutrition. If these compounds are capable of supporting growth responses in microorganisms used to measure the folate content of foods, overestimation of the biologically useful folate content of foods could occur.

#### Assessment of Folate Status

The nutritional status of an individual indicates the degree to which physiological needs for nutrients are being met. Nutritional status is often evaluated using dietary history and intake data, and biochemical and clinical parameters.

Dietary intake. Estimation of the dietary folate content of foods and the evaluation of folate intake by individuals and population groups is complicated by many factors.

Researchers and practitioners need to recognize the limitations of the available folate databases and the problems inherent in collecting dietary intake and food frequency data when using this information to evaluate nutritional status. Examples of problems associated with folate food composition data include: the use of different analytical techniques to determine the folate content of foods; missing information for foods that have not been analyzed; failure to incorporate information on the bioavailability of folate from various foods; and the effect of different methods of food preparation, storage and handling (Bailey, 1990a; Bailey, 1992; Gregory, 1989).

The accurate recording and evaluation of individual dietary intake and food frequency data is also problematic. For example, it is often difficult for individuals to correctly remember the types and/or amounts of foods they have consumed. Food selection may also be affected as a result of being asked to record food intake. Factors such as age, mood, intelligence, attention span, frequency of exposure to the process and perceived importance of the information can also affect the ability to recall and/or record food intake information (Blake et al., 1989; Karvetti and Knuts, 1985; Lissner et al., 1989).

Biochemical and clinical measures of folate status. The progressive changes in biochemical and clinical parameters occurring during folate depletion were determined and

described by Herbert (1962) in a depletion study in which he consumed a folate-deficient diet for four months. As a result of this experiment, Herbert categorized folate depletion into four stages: early negative folate balance; nutrient depletion; biochemical nutrient deficiency; and clinical nutrient deficiency.

During the initial stage of folate depletion, serum folate values become depressed. Serum folate concentrations are very responsive to recent dietary intake, with levels becoming low after consuming a folate deficient diet for only two to three weeks. A value of less than 3 ng/mL is indicative of negative folate balance. Values from 3 to 6 ng/mL represent marginally negative folate balance, with normal serum folate values ranging from 6 to 25 ng/mL. The sensitivity of serum folate to recency of dietary folate intake make it a poor indicator of the degree of folate deficiency. To determine the severity of folate depletion, parameters indicative of body stores and changes in metabolic function need to be measured concurrently (Herbert, 1987; 1990).

The second stage of folate depletion is characterized by a decline in body folate stores. The largest amount of folate is stored in the liver. When folate intake is deficient, normal liver folate stores can be maintained for approximately four months. Coincidentally, the average life span of normal erythrocytes is four months as well. Since the erythrocyte

folate concentration is actually a measure of folate status at the time the erythrocyte was synthesized, erythrocyte folate concentrations usually parallel liver folate stores. This relationship, combined with the greater accessibility of erythrocytes, has resulted in the routine use of erythrocyte folate concentration as a means for determining tissue stores. Thus, stage two is identified by measuring erythrocyte folate concentrations, which decrease to less than 140 ng/mL when tissue stores are low. Erythrocyte folate values between 140 to 160 ng/mL suggest marginal depletion, and concentrations above 160 ng/mL indicate normal folate status (Herbert, 1987; 1990).

Severe depletion of folate stores, characterized by impaired folate-dependent metabolism, represents the third stage of depletion. Folate coenzymes are required for many metabolic functions including the synthesis of thymidylate. A severe folate deficiency retards the synthesis of this nucleotide, and thus interferes with DNA synthesis. Subsequently, deranged DNA synthesis results in morphologic changes in erythrocytes and neutrophils. Neutrophils become hypersegmented because more constriction bands are formed, constricting the DNA into more lobes. Hypersegmentation of neutrophils is identifiable early in the course of impaired metabolism because of the short half-life of these cells. The criterion used to define hypersegmentation of neutrophils is a lobe average equal to or greater than 3.5 lobes per cell

(Herbert, 1987; 1990). This hematologic alteration is thought to be a sensitive screening tool although it is an unreliable indicator of folate status during pregnancy (Herbert et al., 1975) and in a small percentage (1%) of otherwise normal adults with congenital polymorphonuclear leukocyte segmentation (Herbert, 1964).

The morphologic changes manifested in erythrocytes during the third stage of depletion include an increase in size and conversion to an oval shape. At this stage, morphologic damage is confined to the youngest erythrocytes, which are not yet in the majority because of their longer life span, so macroovalocytosis and an increase in the mean corpuscular hemoglobin concentration are not evident. Other changes that occur during stage three of depletion include a further decline in liver and erythrocyte folate concentration (Herbert, 1990).

The final depletion stage is clinically manifested as normochromic, macrocytic anemia. At this point, the majority of the erythrocytes are larger than normal resulting in an increase in the mean corpuscular volume. The hemoglobin level also declines due to decreased erythropoiesis. Further decreases in liver and erythrocyte folate concentrations may also be noted (Herbert, 1990).

The type of anemia caused by a folate deficiency is clinically indistinguishable from that caused by a deficiency of vitamin B<sub>12</sub>. Anemia due to vitamin B<sub>12</sub> deficiency is

thought to be caused by a secondary deficiency of folate. It has been hypothesized that this secondary deficiency develops as a result of trapping folate in the  $N^5$ -methyl-tetrahydrofolate form.

The predominant form of folate in the serum, liver, and most likely, other body storage depots, is  $N^5$ -methyl-tetrahydrofolate. Another source of this form of folate is that which is synthesized from  $N^{5,10}$ -methylene-tetrahydrofolate when control of  $N^{5,10}$ -methylene-tetrahydrofolate reductase is relaxed. Conversion of  $N^5$ -methyl-tetrahydrofolate to tetrahydrofolate is catalyzed by the vitamin  $B_{12}$ -dependent enzyme methionine synthetase. When vitamin  $B_{12}$  is present in adequate amounts, the methyl group from  $N^5$ -methyl-tetrahydrofolate is removed resulting in the regeneration of tetrahydrofolate. This reaction is important for two reasons: 1) there is no other mechanism for regenerating tetrahydrofolate in human cells; and 2) tetrahydrofolate is a precursor to many other folate coenzymes, including  $N^{5,10}$ -methylene-tetrahydrofolate, which is ultimately used for DNA synthesis. According to the "methyl-folate trap" hypothesis (Herbert and Zalusky, 1962), folate gets "trapped" in the  $N^5$ -methyl-tetrahydrofolate form when vitamin  $B_{12}$  is deficient. This form of folate is not usable for any other folate-requiring reactions, including the production of thymidylate. Inadequate production of thymidylate interferes with the synthesis of DNA and eventually causes the development of a



macrocytic anemia that is hematologically identical to that caused by folate deficiency (Herbert, 1990).

The need to properly identify the underlying cause of macrocytic anemia prior to initiating treatment is essential in order to avoid deleterious consequences. For this reason, concurrent assessment of vitamin B<sub>12</sub> and folate status is recommended. As an alternative, the deoxyuridine suppression test can be used to distinguish between macrocytic anemia caused by a deficiency of folate, vitamin B<sub>12</sub> or both of these nutrients. As previously discussed, in the absence of folate and/or B<sub>12</sub> deficiency, deoxyuridine is converted to thymidine by thymidine synthetase. Thymidine is subsequently incorporated into DNA. The deoxyuridine suppression test is an in vitro test that measures the activity of thymidine synthetase by comparing the amount of unlabeled versus labeled thymidine incorporated into DNA, when labeled thymidine is added to bone marrow cells or phytohemagglutinin-stimulated lymphocytes. Labeled thymidine incorporation is suppressed when folate and vitamin B<sub>12</sub> are present in adequate amounts. Conversely, when a deficiency of folate or vitamin B<sub>12</sub> is present, the conversion of deoxyuridine to thymidine is reduced and more labeled thymidine is incorporated into DNA. When suppression of labeled thymidine is low, the underlying cause can be determined by adding N<sup>5</sup>-methyl-tetrahydrofolate to the medium. If folate is deficient, the suppression rate will be increased when this coenzyme form is added. If the

cause is due to a vitamin B<sub>12</sub> deficiency, the addition of N<sup>5</sup>-methyl-tetrahydrofolate will have no effect (Brody, 1991; Herbert, 1990).

In the past, problems associated with sample collection and processing techniques have limited the application of the deoxyuridine suppression test to laboratory research settings. The development of a method using a whole blood (0.1mL) lymphocyte culture instead of cultures of separated lymphocytes has made this test more suitable for use in clinical laboratories, and perhaps, survey studies (Das et al., 1980); however, as a measure of folate status, it is no more informative than erythrocyte folate concentration (Tamura et al., 1990).

The histidine load test is another method that can be used to assess folate status. Formimino-glutamate, a product of histidine catabolism, is further catabolized to glutamate, ammonia and carbon dioxide by tetrahydrofolate formimino-transferase, a folate-requiring enzyme. When folate is deficient, urinary excretion of FIGLU is elevated, with excretion being particularly high after an oral dose of L-histidine. Despite the sensitivity of this test to folate deficiency, it is not specific for folate deficiency since a deficiency of vitamin B<sub>12</sub> will also cause an increase in FIGLU excretion. Additionally, FIGLU excretion is affected by other diseases and physiological conditions, so its application is usually limited to scientific studies (Brody, 1991).

Measurement of plasma homocysteine has recently been proposed as a new method for assessing folate status. Under normal conditions, approximately 50% of the available homocysteine is converted to methionine by the folate-vitamin B<sub>12</sub>-requiring remethylation reaction catalyzed by methionine synthetase (see Figure 2-2). Inhibition of this reaction due to vitamin B<sub>12</sub> deficiency or inborn errors of folate or vitamin B<sub>12</sub> metabolism, results in an accumulation of homocysteine in the blood (Krumdieck, 1990). Kang et al. (1987) and Stabler et al. (1988) investigated the potential association of folate deficiency with homocysteinemia and found a negative correlation between serum folate concentrations and protein-bound homocysteine. Stabler et al. (1988), reported elevated serum homocysteine concentrations in 18 of 19 folate-deficient patients. Compromised folate status was attributed to nutritional inadequacy in 17 of these subjects. However, hyperhomocysteinemia was also found to occur in patients with a deficiency of vitamin B<sub>12</sub> (Stabler et al., 1988), so the total serum homocysteine concentration must be used in combination with other parameters in order to distinguish folate deficiency from a deficiency of vitamin B<sub>12</sub>. Determination of serum methylmalonic acid concentration, which is normal in patients with folate deficiency, has been recommended for this purpose (Stabler et al., 1988).

### Folate Status in Special Population Groups

Folate status is affected by physiological changes occurring at different stages of the life cycle. The potential impact of growth, development and maturation on folate requirements, metabolism, and subsequently, status, in selected population groups, is summarized below.

Infants. The rapid rate of growth that occurs during the first year of life influences folate requirements. Folate requirements are also affected by the developmental immaturity of the infant. For example, low secretion of pancreatic proteases and gastric and biliary secretions during the first months of life may significantly affect the bioavailability of food folate and thereby influence folate requirements and status (Picciano, 1990).

It has been reported that blood folate values of infants at birth are higher than values for pregnant and lactating mothers (Ek and Magnus, 1979) or normal adults (Smith et al., 1985; Vanier and Tyas, 1966), and that these values decline when solid foods are introduced (Smith et al., 1985). A study (Smith et al., 1985) designed to assess the folate status of infants supports the concept that milk alone (human or proprietary formulas) is an important dietary source of folate during the first year of life, and inclusion of this food can provide sufficient folate to maintain blood folate concentrations within an acceptable range.

Preterm, low-birth-weight infants have greater folate requirements than term infants (Rodriguez, 1978). Depressed serum and erythrocyte folate concentrations and megaloblastic anemia have been observed in this population subgroup (Gray and Butler, 1965; Roberts et al., 1969; Strelling et al., 1966). The efficacy of supplemental folate given to low-birth-weight infants was demonstrated by Burland et al. (1971). After comparing the serum and erythrocyte folate values of supplemented infants to those of unsupplemented infants during the first nine months of life, these researchers concluded that folate supplementation should be provided to all low-birth-weight infants, but that more research was needed to determine the optimal route, dose and duration of therapy. Dallman (1974) has stated that folate supplementation at the level of 50  $\mu\text{g}/\text{d}$  for well infants weighing less than 2,000 g at birth is warranted. The Subcommittee on Pediatric Parenteral Nutrient Requirements (Greene et al., 1988) of the American Society for Clinical Nutrition has recommended 56  $\mu\text{g}/\text{kg}/\text{d}$  of folic acid for preterm infants receiving parenteral nutrition.

Adolescents. The rapid growth experienced by adolescents is characterized by increases in lean body mass, skeletal tissue and blood volume. Folate requirements are elevated during this accelerated growth period because of the role folate plays in cell division. Superimposed on the extra demands for folate due to growth, are the effects of other

factors that may adversely affect folate status. These include poor diet, alcohol and drug use, smoking and the possibility of pregnancy in the sexually mature female.

Compromised folate status is prevalent among adolescents, particularly those living in low-income households. Bailey et al. (1982a) reported that 45% of rural black and white adolescent males and females living in low-income households had erythrocyte folate concentrations below 140 ng/mL. Serum folate concentrations were less than 6 ng/mL in 56% of these subjects. A similar trend was found in adolescents from urban low-income households, where 42% of the subjects had erythrocyte folate concentrations less than 140 ng/mL, and 45% had serum folate concentrations below 6 ng/mL (Bailey et al., 1982b). When assessed as a function of sexual maturation, these researchers (Bailey et al., 1982 a; 1982 b) found that serum folate concentrations declined as sexual maturation progressed. A similar relationship was reported by Daniel et al. (1975). Based on erythrocyte folate concentration, Tsui and Nordstrom (1990) found the prevalence of folate deficiency to be 13% among males and 40% among females. In the same study, analysis of seven day food records revealed that for all race, sex and age groups, subjects who were folate deficient had significantly lower folate intakes than those with normal folate concentrations. The type of dietary pattern that contributed to inadequate folate consumption in this study was not addressed, but a study by Bailey et al.

(1984b) suggests that poor folate status among adolescents may be due in part to their limited consumption of vegetables and fruit.

Pregnancy. The RDA for folate is doubled during pregnancy. This large increase in the recommended intake reflects the fact that cell division and multiplication is occurring rapidly. Increased dietary folate, along with increased amounts of other nutrients, are needed to support the physiological and compositional changes occurring at this time. Increased maternal erythropoiesis, uterine and mammary tissue expansion, placental and fetal growth and greater urinary folate losses all contribute to the increased demand for folate during pregnancy (National Academy of Sciences, 1990). During the final weeks of the normal gestational period, the rate of active transport of folate across the placenta is elevated. This places further demands on maternal folate stores and may explain why serum and erythrocyte folate values are usually several-fold higher in neonates than in pregnant and lactating mothers (Bailey, 1990b).

As with other population subgroups, estimates of dietary folate intake during pregnancy by women residing in the United States are limited. A recent study (Huber et al., 1988) of 566 pregnant women who were primarily white, middle class and at least 20 years old, found that only 8.5% of the women derived their folate intake entirely from the diet. The mean folate intake of this group was 257  $\mu\text{g}/\text{d}$ . The remaining women

(91.5%) consumed folic acid supplements and had a mean intake of 1087  $\mu\text{g}/\text{d}$ . The women who did not take folic acid supplements had significantly lower serum and erythrocyte folate concentrations compared to those who used supplements. Data from the 1985 Continuing Survey of Food Intake (United States Department of Agriculture, 1987) indicated that the mean folate intake by women (nonpregnant) between the ages of 19 and 34 (all income levels) was 217  $\mu\text{g}/\text{d}$ . These data suggest that unless women are motivated to make dietary changes, or are instructed or knowledgeable enough to recognize the need to take a folic acid supplement during pregnancy, the recommended allowance for folate may not be met. This is particularly disconcerting in light of the protective effect of folate against neural tube defects (Anon., *Morbidity and Mortality Weekly*, 1992).

A high prevalence of folate deficiency has been suggested by population studies of pregnant women in whom blood folate concentrations were measured. Herbert et al. (1975) studied 110 low-income, predominantly black or Puerto Rican women living in New York City. These researchers reported that 20% of the subjects had serum folate values below 3 ng/mL, and 16% of the subjects had erythrocyte folate values below 150 ng/mL. Bailey et al. (1980) analyzed blood samples from low-income women in Florida and found that 29% had erythrocyte folate values below 140 ng/mL. Bailey did not attribute the low erythrocyte folate concentrations found in these studies to



hemodilution because erythrocyte folate concentrations are indicative of the folate available to precursor red cells in the bone marrow at the time the currently circulating cells were developed (Bailey, 1990b).

The value of folic acid supplementation in the prevention of folate deficiency during pregnancy was demonstrated in a study of African women (Colman et al., 1975). Pregnant women receiving folic acid supplemented cereals experienced significant increases in serum and erythrocyte folate concentrations, whereas the erythrocyte concentrations in unsupplemented women decreased by an average of 42 ng/mL during the last month of gestation (33 days). Despite a hemoglobin concentration of 11 g/dL or more at the start of the study, the supplemented women experienced an increase in hemoglobin concentration suggesting that hematopoiesis was limited by folate deficiency. These researchers suggested that this finding gives further credence to the need for folic acid supplementation in this population.

Not all researchers agree that low serum folate concentrations during pregnancy are associated with maternal complications or congenital malformations of the fetus (Hall et al., 1976). For example, Hall et al. (1976) examined over 2700 women at four time points and found progressive reductions in serum folate values at each stage. Maternal complications and fetal outcome were not reported for any of these pregnancies. Women from low socioeconomic groups,

smokers, multigravidae and women with twin pregnancies had greater declines in serum folate. With the exception of the smokers, however, significant reductions in mean serum folate concentrations were not detected. It was concluded that this decline was due to plasma volume expansion and did not warrant routine folate supplementation.

Lactation. The effect of lactation on maternal and infant status and milk folate content in unsupplemented women has been described by several researchers. Smith et al. (1983) found that blood folate concentrations were lower in well-nourished, unsupplemented lactating women compared to supplemented lactating women and normal nonlactating controls. While the erythrocyte folate concentration of the unsupplemented women declined, the folate content of their milk was comparable to that of the supplemented women. There was no difference in the blood folate concentrations of infants of supplemented or unsupplemented mothers. Similar results were found in a study described by Metz (1970). In this study, lactating women fed a controlled low-folate diet experienced rapid reductions in serum folate concentrations, while the folate content of their milk remained constant.

When folate status during pregnancy and lactation is severe enough to cause megaloblastic anemia, oral administration of supplemental folic acid to lactating mothers has been shown to improve their milk folate content (Cooperman et al., 1982). Milk folate concentrations have also been

increased in lactating women of low socioeconomic status when folic acid supplements were administered (Sneed et al., 1981). However, serum folate concentration appears to increase, while milk folate content remains constant when maternal blood folate levels are already within an acceptable range (Tamura et al., 1980). These findings suggest that a regulatory mechanism controls the level of milk folate secretion (Tamura et al., 1980) with the concentration of folate in breast milk being maintained at the expense of maternal reserves and status (Smith et al., 1983). Folic acid supplementation during pregnancy and lactation may help to protect the folate status of lactating women (Smith et al., 1983).

Elderly. While some researchers (Baker et al., 1978) have suggested that age related changes adversely affect folate absorption, other investigators (Bailey et al., 1984a) have concluded that folate absorption is not affected by the aging process. Although alterations in other aspects of folate metabolism may exist, as intimated by data showing depressed erythrocyte folate uptake in elderly subjects (Ettinger and Colman, 1985), assessment studies of population subgroups of the elderly suggest that socioeconomic and environmental factors are probably the most important contributors to the development of compromised folate status.

The potential effect of socioeconomic level on folate status in the elderly population is apparent when comparing assessment studies conducted in population subgroups with

different income levels. The majority of high-income elderly subjects participating in studies conducted in New Mexico (Garry et al., 1984) and Florida (Wagner et al., 1981) had normal serum and erythrocyte folate concentrations. Only 3% and 6% of the participants in the New Mexico and Florida studies, respectively, had erythrocyte folate values below 140 ng/mL. Conversely, Bailey et al. (1979) found that 60% of elderly Floridians from very poor socioeconomic backgrounds had erythrocyte folate concentrations below 140 ng/mL, as well as evidence of macrocytic anemia.

Environmental factors that may place the elderly at higher risk for the development of compromised folate status have been reviewed by Bailey (1990b) and Sauberlich (1990). These factors include: institutionalization; chronic use of prescription and/or nonprescription medications; the presence of disease; and the consumption of alcohol.

Although not specifically addressed for each stage of the life cycle covered above, it is important to consider the impact that cultural food habits and customs, food preferences, educational level, state of mental and physical health, socioeconomic factors and food availability have on food selection and nutrient intake. While physiological changes occurring throughout the life cycle can have a profound affect on nutrient needs and metabolism, Sauberlich (1990) has concluded that the most common cause of compromised folate status is inadequate dietary intake.

### Effects of Environmental Factors on Folate Status

Prescription and nonprescription medications. Certain prescription and nonprescription medications interfere with folate absorption and/or metabolism, and depending on the dose and duration of use, may result in compromised folate status. Examples of these drugs include folate antagonists, anticonvulsants, histamine receptor blockers, antacids, anti-inflammatory agents, aspirin and possibly oral contraceptive agents.

Folate antagonists are used in cancer chemotherapy or to treat infections such as malaria. Methotrexate is an example of a folate antagonist. This drug interferes with folate metabolism by inhibiting dihydrofolate reductase, resulting in a functional folate deficiency and anemia. Methotrexate can also cause reversible mucosal ulceration (Roe, 1989). The resulting damage to enterocytes can impair the absorption of folate and other nutrients.

Rosenberg et al. (1982) have noted that the frequent use of antacids and histamine receptor blockers among the elderly poses a theoretical risk to this population in terms of their folate status. Histamine receptor blockers such as cimetidine, and antacids like sodium bicarbonate, may impair folate status by increasing the intestinal pH to levels that exceed the optimum for folate conjugase activity and carrier-mediated transport and/or passive diffusion (MacKenzie and Russell, 1976; Russell et al., 1979). However, folate

bioavailability was not reduced by chronic bicarbonate administration in a rat bioassay (Hoppner and Lampi, 1988). Furthermore, evidence of folate deficiency due to use of these medications is lacking (Rosenberg et al., 1982).

Sulfasalazine is an anti-inflammatory agent frequently used in the treatment of inflammatory bowel disease. Franklin and Rosenberg (1973) have demonstrated that this drug interferes with folate absorption. It appears that sulfasalazine interferes with the absorptive process through competitive inhibition (Strum, 1981), and by inhibition of jejunal brush border folate conjugase activity (Reisenauer and Halsted, 1981). Sulfasalazine also has the potential to disturb folate metabolism since it can inhibit several hepatic folate-dependent enzymes (Selhub et al., 1978). Patients taking daily therapeutic doses of sulfasalazine are at risk of developing folate deficiency and should be provided with therapeutic doses of folic acid in order to prevent a deficiency of this nutrient.

The interaction between folate and diphenylhydantoin, an anti-convulsant medication used for treatment of epilepsy, may actually be a two-way interaction (Reynolds, 1973; Rivey et al., 1984). Chronic use of this drug has been associated with folate deficiency and anemia, although the progression of the deficiency to megaloblastic anemia is rare (Gerson et al., 1972; Hoffbrand and Necheles, 1968; Reynolds, 1973; Rivey et al., 1984; Rosenberg et al., 1968) and administration of

phenytoin has not been shown to affect the kinetics of folate excretion (Krumdieck et al., 1978). Conversely, long-term, high-dose folic acid supplementation of folate-deficient patients taking diphenylhydantoin may result in lowered serum concentrations of this drug in selected patients, with the potential for loss of control of the seizure disorder (Reynolds, 1973; Rivey et al., 1984).

The mechanism responsible for the development of compromised folate status in patients receiving diphenylhydantoin is unclear, but several hypotheses have been espoused. These hypotheses have been reviewed by Rivey et al. (1984). They include: drug inhibition of brush border folate conjugase (Hoffbrand and Necheles, 1968; Rosenberg et al., 1968); elevation of the intraluminal pH resulting in malabsorption of dietary folate (Benn et al., 1971); drug-induced impairment of folate transport into tissues (Krumdieck et al., 1978); and induction of folate-requiring metabolic processes in the liver (Maxwell et al., 1972). Studies employed to prove and/or disprove each of these hypotheses have provided seemingly contradictory results. Rivey et al. (1984) have suggested that diphenylhydantoin may affect folate homeostasis by multiple mechanisms.

Alter et al. (1971) and Lawrence et al. (1984) have reported that aspirin in therapeutic doses can reduce serum folate concentrations. After aspirin is discontinued, serum folate values rapidly increase. Individuals with rheumatoid

arthritis, as well as those taking therapeutic doses of aspirin for the prevention of heart attacks, may be particularly vulnerable to the effects of chronic aspirin ingestion. Elderly individuals are more likely to use aspirin for these purposes, which may make them more susceptible to folate depletion depending on the amount and frequency of use.

Several investigators have noted an association between the use of oral contraceptive agents and low serum and erythrocyte folate values (Butterworth et al., 1982; Pietarinen et al., 1977; Smith et al., 1975). Other researchers have found no difference in folate status of oral contraceptive users versus nonusers (Paine et al., 1975; Ross et al., 1976; Whitehead et al., 1973). Results of studies comparing blood folate concentrations of women using oral contraceptive agents within six months of conception to those of nonusers have also been equivocal (Bailey, 1980; Martinez and Roe, 1977). Factors such as the specific formulation of the pill (i.e. level of estrogen), duration of pill use and/or dietary intake may affect the body's response to oral contraceptive agents. Based on observations that megaloblastic changes in the cervical epithelium of women taking oral contraceptive agents were reversed with oral folic acid supplements (Whitehead et al., 1973), some researchers have speculated that oral contraceptive use may lead to a



localized folate deficiency in the cervix (Butterworth et al., 1982).

Smoking. The effects of smoking in nonpregnant women were investigated by Wittier et al. (1982). The mean serum and erythrocyte folate concentrations of the smokers who participated in this study were lower than those of the nonsmokers. No differences between smokers were detected, however, with regard to the number of cigarettes smoked per day.

Heimbürger et al. (1987; 1988) compared the folate status of male smokers to that of a control group of nonsmokers and found significantly lower serum and erythrocyte folate concentrations in the smokers. Smokers with metaplasia had lower blood folate concentrations than did smokers without metaplasia, and folate concentrations appeared to decrease with increasing severity of metaplasia. These studies suggest that smoking may adversely affect folate status.

Alcohol. Compromised folate status is common in chronic alcoholics and is probably caused by a combination of factors including: poor dietary habits; intestinal malabsorption; decreased hepatic uptake; and increased urinary folate excretion (Halsted, 1980). The effects of binge drinking on folate absorption have been illustrated by studies using orally administered radio-labeled folic acid. Evidence of folic acid malabsorption was provided by decreased plasma concentrations of radioactivity after oral doses of tritiated

pteroylmonoglutamic acid ( $[^3\text{H}]\text{PteGlu}$ ) (Halsted et al., 1967) and by reduced luminal disappearance of  $[^3\text{H}]\text{PteGlu}$  during jejunal perfusion (Halsted et al., 1971). The underlying defect responsible for the limited jejunal uptake of folic acid was thought to be due to inadequate intake of dietary folate during chronic alcohol ingestion (Halsted et al., 1971). As discussed by Halsted (1990), a subsequent human study that examined the effects of a folate deficient diet on the absorption of  $[^3\text{H}]\text{PteGlu}$  (Halsted et al., 1973), and an absorption study conducted with monkeys fed diets containing 50% of their calories as ethanol (Romero et al., 1981), prompted researchers to hypothesize that folate malabsorption in chronic alcoholism results from the combined effects of folate deficiency and ethanol exposure.

Recent research has attempted to delineate the pathogenesis of folate malabsorption occurring in alcoholics. To separate the effects of poor diet from those of alcohol exposure, studies using miniature pigs exposed to alcohol for a short period of time and in the absence of folate deficiency have been performed (Naughton et al., 1989; Reisenauer et al., 1989). These studies suggest that inhibition of brush border folate conjugase, resulting in decreased hydrolysis of folate polyglutamates, may be the earliest functional lesion contributing to folate malabsorption and deficiency in alcoholism (Halsted, 1990).

Disease. Folate deficiency has been associated with a variety of diseases including certain hemolytic diseases (Brody, 1991), cancer of the head and neck (Brody, 1991), inborn errors of metabolism (Brody, 1991) and diseases of the intestinal mucosa (Corcino et al., 1976; Halsted et al., 1977; 1978; Hoffbrand et al., 1968; 1970). The proposed underlying etiology of folate deficiency in these diseases is defective absorption and/or altered folate metabolism. These defects lead to an increased folate requirement. For example, the increased damage to red blood cells occurring in hemolytic diseases results in increased cell division in the bone marrow. This increase in cell division is thought to elevate the requirement for folate (Brody, 1991). Altered metabolism may also be responsible for the folate deficiency that develops in patients with certain types of cancer, particularly cancer of the head and neck (Brody, 1991). The effects of altered folate metabolism in these individuals is independent of the effects of anticancer drugs such as methotrexate which can also adversely affect folate status. Patients with inborn errors of folate metabolism may have lower levels of folate-dependent enzymes, as well as defective folate absorption, thereby increasing their folate requirement (Brody, 1991).

Malabsorption is thought to be the major defect contributing to compromised folate status associated with untreated gastrointestinal diseases such as celiac sprue and

tropical sprue. Luminal disappearance of [ $^3\text{H}$ ]PteGlu and pteroyl [ $^{14}\text{C}$ ]-glutamylhexaglutamate ([ $^{14}\text{C}$ ]-PteGlu<sub>7</sub>) in patients with celiac sprue (Halsted et al., 1977; 1978) and tropical sprue (Corcino et al., 1976) is significantly less than luminal disappearance in normal subjects. When appropriate medical and nutritional management of these diseases is instituted, luminal disappearance increases significantly (Corcino et al., 1976; Halsted et al., 1977; 1978). Further evidence corroborating the idea that malabsorption adversely affects folate status, at least in terms of celiac sprue, comes from two studies. A significant decrease in the hydrolysis of perfused [ $^{14}\text{C}$ ]-PteGlu<sub>7</sub> in patients with celiac sprue was noted in one study (Halsted et al., 1977). The other experiment showed that brush border folate conjugase activity in jejunal biopsy specimens taken from patients with celiac sprue was significantly lower than the activity of this enzyme in specimens from normal subjects (Halsted et al., 1986).

### Consequences of Compromised Folate Status

Megaloblastic anemia is probably the most commonly recognized clinical problem associated with folate deficiency, but research is beginning to identify many new potential consequences of compromised folate status. Negative outcomes more recently associated with folate deficiency include neural tube defects, compromised infant birth weight, increased

potential for neoplastic changes and altered immune function. A brief review of the evidence linking folate deficiency to these problems is presented in this section.

Neural tube defects. Neural tube defects, which include spina bifida, anencephaly and encephalocele, are among the most common severe congenital malformations. The idea that folate deficiency contributed to the causation of fetal malformations was introduced almost thirty years ago by Hibbard (1964) and Hibbard and Smithells (1965). Smithells et al. (1980; 1981) conducted the first intervention trial which suggested that supplementation with folic acid (0.36 mg/d) or other vitamins near the time of conception might reduce the risk of recurrence of birth defects categorized as neural tube defects. The results of this study could not be taken as definitive proof of a protective effect for folate, however, due to the co-administration of folic acid with other vitamins, the lack of randomization of subjects to the control and treatment groups and the absence of a double-blind design.

Another early intervention trial was conducted by Laurence et al. (1981). This was a small study that employed a controlled, randomized, double-blind design to test the effects of folic acid supplementation (4 mg/d) alone. The data provided inconclusive results when analyzed using the original randomization scheme; however, when the data for women who did not take their supplements were transferred to the control group, the supplemented subjects had a

significantly lower recurrence rate. Preliminary results of a study using a similar design and a small number of subjects, found a reduction in the recurrence rate after a supplementation program had been initiated, compared to the recurrence rate before a supplementation program had been established (Holmes-Siedle et al. 1982).

These early studies supported the concept of a protective role for folic acid, but their flawed designs precluded the ability to make any definitive conclusions and recommendations. The results of a recent study (Medical Research Council (MRC) Vitamin Study Research Group, 1991), however, have established that supplementation with folic acid around the time of conception can decrease the risk of neural tube defects in women who have previously given birth to an affected infant. This study was a large, multi-center, double-blind intervention trial that randomized participants to one of four groups: 1) folic acid alone; 2) folic acid plus other vitamins; 3) other vitamins alone; and 4) no supplements. This design allowed the researchers to determine if vitamins other than folic acid conferred a protective effect. The results demonstrated that folic acid, rather than the combination of other vitamins, was responsible for the improved outcome.

While the MRC Vitamin Study Research Group (1991) findings are positive with respect to folate's role in reducing the risk of recurrent neural tube defects, they do

not address the association of folate intake with the occurrence of neural tube defects. In the United States, most (approximately 95%) of the neural tube defect-affected infants and fetuses occur in pregnancies of women who have not previously given birth to an infant with a neural tube defect. Thus, in order to reduce the overall prevalence of neural tube defects, folic acid supplementation must exert a protective effect on the developing embryos of women who have no history of a neural tube defect-affected pregnancy. The MRC study also leaves open to question the minimum dose and form of folate needed to confer a protective effect, since subjects were given daily supplements containing 4 mg of folic acid.

Over the last five years, several observational studies have been conducted to evaluate the impact of periconceptional folic acid/vitamin supplementation on the occurrence of neural tube defects. Three of these studies were case-control studies (Bower and Stanley, 1989; Mills et al., 1989; Mulinare et al., 1988) and one was a prospective cohort study (Milunsky et al., 1989). All of these studies involved women who had no history of a neural tube defect-affected infant/fetus. The occurrence of neural tube defects in women who reported taking multivitamins containing folic acid (approximately 0.4-0.8 mg folic acid per day) for at least one month prior to conception through the first trimester of pregnancy were compared to women who did not take supplements. Dietary intake of folate was also considered in some studies. A protective effect was

associated with multivitamin supplement use and/or higher levels of dietary folate intake in three (Bower and Stanley, 1989; Milunsky et al., 1989; Mulinare et al., 1988) of the four studies.

Two new studies have provided additional support in favor of a protective effect of folate. One study was a randomized controlled trial conducted in Hungary (Czeizel and Dudás, 1992). Subjects were 18-35 years old, were not pregnant at the time of recruitment and had no history of infertility or fetal death. The volunteers were randomized to receive either a placebo or a multivitamin supplement (including 0.8 mg folic acid). There were no cases of neural tube defects in the 2104 participants receiving the multivitamin, whereas, the placebo group had six occurrences and 2046 unaffected pregnancies. Although the criteria for inclusion did not specifically exclude women with a history of a neural tube defect-affected pregnancy, it is likely that most women falling into this category would have already been recruited for the MRC study and would not have been available to participate in the study just described. Under this assumption, these results have been interpreted as providing evidence that folic acid can reduce the risk of occurrence of neural tube defects; and that the quantity of folic acid needed to produce such an effect is much less than the 4 mg daily dose used in the MRC trial.

The results of another study examining the rate of occurrence of neural tube defects in folate supplemented and



unsupplemented women has recently been published (Werler et al., 1993). This large case-control study was conducted in Boston, Philadelphia and Toronto. The control group consisted of 2615 infants with birth defects other than neural tube defects and oral clefts. This group was compared to 443 cases with neural tube defects. The prevalence of use of folic acid-containing multivitamins during the periconceptional period was compared between mothers of cases and controls. Daily folic acid supplementation was found to reduce the risk of occurrent neural tube defects by 60%. Since the most commonly used dose of folic acid was 0.4 mg, these data were considered to be consistent with the hypothesis that 0.4 mg supplemental folic acid per day is sufficient to decrease the risk of neural tube defects among pregnancies of women in the general population.

In the wake of the findings of the MRC trial (1991) and the studies by Czeizel and Dudás (1992) and Werler et al. (1993) the data from observational studies have been reevaluated. Taken together, the results have been interpreted as providing support for the hypothesis that folic acid will decrease the risk of occurrence, as well as the recurrence, of neural tube defects. Accordingly, the United States Public Health Service has recently recommended that:

All women of childbearing age in the United States who are capable of becoming pregnant should consume 0.4 mg of folic acid per day for the purpose of reducing their risk of having a pregnancy affected with spina bifida or other NTDs. Because the effects of higher intakes are not well known but include complicating the diagnosis of vitamin

B<sub>12</sub> deficiency, care should be taken to keep total folate consumption at <1 mg per day, except under the supervision of a physician. Women who have had a prior NTD-affected pregnancy are at high risk of having a subsequent affected pregnancy. When these women are planning to become pregnant, they should consult their physicians for advice. (Anon., *Morbidity and Mortality Weekly*, 1992, p. 1)

Recognition of the importance of folate in reducing the risk of bearing a child with a neural tube defect is certain to stimulate research focused on understanding the mechanism whereby folate exerts its protective effect. Particular attention is likely to be directed at identifying the existence of underlying defects in folate metabolism.

Compromised birth weight. Folate deficiency occurring during pregnancy has been associated with low infant birth weight. Several researchers have reported increased infant birth weights when folic acid supplements were given to folate-deficient pregnant women (Baumslag et al., 1970; Iyengar and Rajalakshmi et al., 1975; and Rolschau et al., 1979). Placental weights were measured in two of these studies (Iyengar and Rajalakshmi et al., 1975; Rolschau et al., 1979), and a positive association with birth weight was noted. These findings suggest that folic acid supplementation improves fetal outcome by improving nutrition via increased placental size (Bailey, 1990b). Additional evidence suggesting a positive relationship between folic acid supplementation and infant birth weight has recently been published by Goldenberg et al. (1992). These researchers found that maternal serum folate concentrations, occurring

within a range suggestive of compliance with the supplementation regimen, were associated with higher infant birth weight and a decreased rate of fetal growth retardation.

Neoplastic changes. The potential relationship between folate and cancer was identified as early as 1944 when several researchers noted that large doses of folic acid directly interfered with the growth of certain tumors. Subsequently, it was found that administration of folate antagonists produced beneficial effects in patients with certain forms of cancer. This discovery, although it revolutionized the treatment of childhood leukemia and other forms of cancer, probably contributed to the paucity of further research regarding the potential anticancer effect of folate (Butterworth, 1991). Renewed interest in the anticancer potential of folate has been sparked by recent findings suggesting that inadequate dietary intake of folate and/or compromised folate status may be associated with an increased risk or prevalence of certain forms of cancer; and that optimal folate intake may provide a protective effect.

The initiation of carcinogenic activity is thought to occur due to altered regulation of genetic expression of both endogenous and exogenous oncogenes. Although the role of folate deficiency in carcinogenesis has not been completely elucidated, the co-carcinogenic effect of a deficiency of this nutrient is thought to be related to folate's role in the maintenance of chromosome structure and function. It has been

well established that folate is important in the regulation of methyl groups used for DNA methylation. A deficiency of methyl groups due to folate deficiency results in undermethylated DNA and substitution of uridylate for thymidylate. Subsequently, this interferes with histone binding and results in the transcription of genetic sequences that would ordinarily be suppressed. Additionally, improper histone binding results in increased exposure of DNA to attack by endogenous nucleases, thereby raising the risk of chromosome breaks and incorporation of viral genomes. Chromosome breaks associated with folate deficiency occur at specific heritable fragile sites and many folate-sensitive breaks occur at positions known to be associated with translocations seen in cancer. It is thought that these translocations disturb regulatory patterns of contiguous segments of genetic information (Butterworth, 1991; Eto and Krumdieck, 1986).

Compromised folate status has been associated with increased risk of dysplasia or cancer of the cervix, colon, bronchus and esophagus. The research documenting these associations is briefly reviewed in the following paragraphs.

The concept of "localized folate deficiency" was introduced as a result of a study conducted by Whitehead et al. (1973). These researchers reported the occurrence of megaloblastic features in cervical epithelial cells from women taking oral contraceptive agents. These cytologic

abnormalities were not related to hematologic changes or low serum folate/vitamin B<sub>12</sub> concentrations; however, after three weeks of folic acid supplementation (10 mg/d), the abnormal cytologic findings were reversed or improved. Later, Butterworth et al. (1982) reported that daily oral folic acid supplementation (10 mg/d) was associated with improvement in the cytologic manifestations of dysplasia compared with placebo-treated controls. The supplemented group also had less severe biopsy readings after three months of supplementation. On the basis of these findings, it was suggested that folate deficiency either plays an integral role in the dysplastic process or is occasionally misdiagnosed as cervical dysplasia (Butterworth et al., 1982).

In a more recent study, Butterworth et al. (1992b) examined the effect of high-dose oral supplements of folic acid (10 mg/d for six months) on the course of cervical dysplasia. Supplementation appeared to have no significant effect on the course of established cases of dysplasia, with a high rate of apparent regression occurring in both the placebo-treated and the folic acid-supplemented groups. These researchers attributed the difference between the findings of this study and their earlier report (Butterworth et al., 1982) to the use of a more adequate sample size, exclusion of patients with atypia less than dysplasia and a longer period of observation. Although they concluded that folic acid supplementation does not alter the course of established

disease, they did not exclude the possibility that folate deficiency played a role in carcinogenesis because they found a higher prevalence of dysplasia associated with human papillomavirus 16 (HPV-16) infection (an oncogenic strain thought to cause cervical dysplasia) among women in the lower two tertiles of red blood cell folate than in the highest tertile. Based on this information, they concluded that folate deficiency may act as a co-carcinogen during the initiation of cervical dysplasia.

In a separate case-control study conducted by the same research group (Butterworth et al., 1992a), infection with HPV-16 was the strongest risk factor for cervical dysplasia, and there was a statistically significant interaction between low erythrocyte folate concentrations and the HPV-16 virus. The conclusion reached by these investigators was that the carcinogenic effect of HPV-16 infection is enhanced in women with low concentrations of erythrocyte folate. Although the mechanism of interaction between folate and HPV-16 was not investigated in this study, the researchers suggested that a folate deficiency might increase the possibility of incorporating the viral genome into human DNA resulting in transformation of the epithelial cells.

The potential role of folate deficiency in carcinogenesis may not be limited to the cervix since premalignant lesions occurring in other organs exhibit features similar to those seen in cervical dysplasia (Heimbürger et al., 1987). Studies

examining the relationship between folate status and bronchial metaplasia suggest that folate deficiency may influence the susceptibility of the bronchial mucosa to neoplastic transformation. For example, Heimbürger et al. (1987) found that the serum and erythrocyte folate concentrations of men who smoked an average of 25 cigarettes per day were significantly lower than nonsmokers, with the lowest values occurring in smokers with bronchial metaplasia. Serum and erythrocyte folate concentrations were also significantly lower in smokers with metaplasia compared to smokers without metaplasia. In a subsequent double-blind intervention trial (Heimbürger et al., 1988), smokers with bronchial squamous metaplasia were stratified according to smoking level and randomly assigned to treatment with a placebo or 10 mg of folic acid and 500  $\mu$ g of hydroxycobalamin for four months. Reduction of atypia, as determined by direct cytological comparison, was significantly greater in the supplemented group. Unfortunately, it is impossible to determine if folic acid alone was responsible for the favorable effect since hydroxycobalamin was administered simultaneously. Nevertheless, these studies are provocative and provide fertile ground for continued investigation in this area.

Compared with the general population, patients with chronic ulcerative colitis are at greater risk for developing cancer of the colon. It has been hypothesized that folic acid supplementation may protect against the development of

dysplasia (a premalignant pathologic finding) and cancer in ulcerative colitis. To test this hypothesis, Lashner et al. (1989) conducted a case control study to examine the effect of folic acid supplementation on the incidence of dysplasia or cancer in 99 subjects with chronic ulcerative colitis. A 62% lower incidence of neoplasia was associated with folic acid supplementation compared to subjects not receiving supplementation. Although this outcome was not statistically significant, it did not change when adjustments were made for known confounders, suggesting that this finding was not due entirely to bias or confounding and that inadequate sample size may have contributed to the lack of significance (Lashner et al., 1989).

Folate deficiency may also play a role in the development of esophageal cancer. Jaskiewicz et al. (1988) have examined cytological specimens obtained by brush biopsy of the esophagus from subjects living in an area of Africa where squamous cell carcinoma of the esophagus is the most common form of cancer. Biopsy results revealed morphologic features similar to those seen with folate deficiency and dysplasia. Assessment of folate status in this same population revealed significantly lower erythrocyte folate concentrations in subjects with dysplastic and cancer cells compared with controls (van Helden et al., 1987). Additionally, the erythrocyte folate concentrations of subjects living in high-incidence districts was significantly lower than those in the



intermediate and low incidence districts. However, these results must be interpreted cautiously since multiple nutrient deficiencies, particularly in the high-incidence area, were noted.

Altered immune function. Although the effect of compromised folate status on immunocompetence has not been widely researched, studies using folate-deficient animals and observations of patients with megaloblastic anemia due to folate deficiency suggest that alterations occur in both humoral and cell-mediated immunity. This is not surprising, since folate is essential for the synthesis of DNA, and a deficiency of this nutrient would hinder the ability of the sensitized cells to proliferate rapidly. Examples of the effects of compromised folate status on immune function as determined from human studies include delayed cutaneous hypersensitivity and depressed peripheral lymphocyte response to phytohemagglutinin. Similar changes in immune function have been shown using animal models. In addition to these changes, reductions in white blood cells, leukocytes, granulocytes, antibody forming cells and T-cells have been reported in various animal models subjected to an isolated folate deficiency (Nauss and Newberne, 1981).

#### Methods Used to Assess Folate Status and Metabolism in Humans

Microbiological assay. The microbiological assay is considered the most useful method for quantifying folate

levels in biological samples (Tamura, 1990). This method uses the growth response of folate-sensitive microorganisms to determine folate content. The assay is performed by comparing the growth of a test organism added to folate-free media containing an aliquot of the sample, to the growth of the same organism added to folate-free media that has been enriched with known concentrations of folic acid. Growth is measured as turbidity following incubation under controlled conditions. The microorganism used most often for this assay is *Lactobacillus casei*, although *Streptococcus faecium* or *Pediococcus cerevisiae* may also be used.

The three species of bacteria that may be used for this assay do not respond equally to the various forms of folate. For example, *S. faecium* does not respond to N<sup>5</sup>-methyl-tetrahydrofolate, nor to folate derivatives with more than two molecules of glutamate. *Pediococcus cerevisiae* is even more selective. It does not respond to N<sup>5</sup>-methyl-tetrahydrofolate, and of the remaining folate derivatives, it can only use those occurring as reduced monoglutamyl tetrahydrofolates (Tamura, 1990). Since neither *S. faecium* nor *P. cerevisiae* respond to N<sup>5</sup>-methyl-tetrahydrofolate, they are not suitable test organisms for determination of serum or erythrocyte folate concentration. *Lactobacillus casei*, however, responds to N<sup>5</sup>-methyl-tetrahydrofolate, as well as other folate derivatives, which makes it the best choice when analyzing biological samples for total folate.

The fact that *L. casei*, *S. faecium*, and *P. cerevisiae* respond differently to folates with varying chain lengths, one-carbon substituent groups and oxidation states offers researchers the opportunity to estimate the amount of folate derivatives present in a particular sample. This can be useful when the quantity of sample available for analysis is insufficient to perform column chromatography. For example, if it is important to estimate the amount of folate derivatives other than N<sup>5</sup>-methyl-tetrahydrofolate, *L. casei* can be used in combination with *S. faecium*. Similarly, *P. cerevisiae* can be used in conjunction with *L. casei* to estimate folate derivatives other than N<sup>5</sup>-methyl-tetrahydrofolate and oxidized pteroylglutamic acid. This deductive approach can also be used to determine whether samples contain folate polyglutamates. If the activity of *S. faecium* or *P. cerevisiae* increases after folate hydrolase treatment, the sample contains folate polyglutamates with more than two molecules of glutamate. Likewise, if *L. casei* activity increases after enzyme treatment, the sample contains polyglutamates with more than three molecules of glutamate (Tamura, 1990).

Biological samples typically analyzed for folate content include serum, whole blood, urine and tissues. Cellular folates, such as those found in tissues, are usually in the polyglutamate form and require cleavage to the monoglutamate form before folate concentration using the microbiological

assay can be determined. Partially purified folate hydrolase preparations from chicken pancreas, hog kidney and rat or human plasma have been used for this purpose. Folate in the erythrocytes is also in the polyglutamate form; however, the presence of natural folate hydrolase in the blood precludes the need for treating hemolyzed whole blood samples with an exogenous folate hydrolase. Urine and serum samples do not require prior treatment with folate hydrolase because the folate present in these samples is in the monoglutamyl form.

Although the basic concept of the microbiological assay has not changed over the years, several improvements have been made. Examples of these improvements have been reviewed by Tamura (1990) and include the addition of antioxidants (e.g. ascorbic acid) to samples to protect labile reduced forms of folate from oxidation and the use of a cryoprotected organism to maintain constant growth-response curves. Recently, this assay has been adapted to take advantage of the availability of the 96-well microtiter plate reader (Horne and Patterson, 1988; Newman and Tsai, 1986; O'Broin and Kelleher, 1992). Although the procedure followed with this adaptation is similar in concept to the standard procedure, the microtiter plate reader improves the efficiency of absorbance readings. Rapid calculation of the results can be achieved by interfacing the microtiter plate reader with a computer (Horne and Patterson, 1988). These modifications can save a

tremendous amount of time and money without sacrificing accuracy (Newman and Tsai, 1986; O'Broin and Kelleher, 1992).

Radiometric binding assay. The radiometric binding assay is a competitive protein binding assay in which radio-labeled folate and unbound folate in the sample compete for the binding sites on folate binding proteins. This method is usually performed by clinical laboratories using commercially available radioassay kits (Brody, 1991; Tamura, 1990).

Compared with the microbiological assay, this competitive binding assay is simpler to perform and is not affected by bacterial contamination or the presence of antibiotics. Despite these potential advantages, the usefulness of the radiometric binding assay is limited because the proteins used to bind folates do not have an equal affinity for all forms of folate. Binding affinity seems to be influenced by the state of oxidation, the one-carbon substituent group and glutamyl chain-length. For this reason, the suitability of the radioassay method for quantifying complex mixtures of naturally occurring folates has been questioned. This method may be acceptable for determining serum folate concentration, however, because N<sup>5</sup>-methyl-tetrahydrofolate is the predominant form of folate in serum, and this form of folate is tightly bound by folate binding proteins. Conversely, if the population being assessed is receiving large doses of folates other than N<sup>5</sup>-methyl-tetrahydrofolate, the radioassay may be inappropriate (Brody, 1991; Tamura, 1990).

Folate bioavailability studies. Until recently, most of the information known about the metabolic requirements and bioavailability of folates in humans was derived from animal bioassay studies, or from a limited number of human studies which used plasma or urinary folate concentration in folate-saturated human subjects as the response criteria. While these studies have made an important contribution to our understanding of folate metabolism, they have several limitations which restrict their scope and applicability. A potential limitation of animal bioassay studies is the use of animal models that are not entirely appropriate for studying human metabolism. For example, rats are not appropriate models to use for folate polyglutamate absorption studies because they exhibit little or no brush border folate conjugase activity (Wang et al., 1985). Limitations of bioassay studies conducted in human subjects include their inability to provide information about in vivo metabolism and the need to consume large quantities of tested foods or purified folates, the latter of which could change the rate of digestion and absorption in a way that is different from that which usually occurs (Gregory and Toth, 1990).

The development of new tools and techniques combined with previous accomplishments has facilitated research in the area of folate requirements and bioavailability. The synthesis of various forms of folate, including folate polyglutamates (Krumdieck and Baugh, 1969; Godwin et al., 1972), and the

ability to radio-label folate derivatives with tritium at different locations in the molecule (Godwin et al., 1972), provided valuable tools for initial studies of folate metabolism in animals. Although radio-labeled folates have been used in human studies, concern about their safety precluded their use for long-term supplementation studies and restricted the amount of labeled folate that could be administered to no more than a tracer dose. The development of stable-isotopically (deuterium) labeled folates (Gregory, 1990; Gregory and Toth, 1988a; 1988b) has circumvented these problems, while providing the opportunity to trace the fate of folate during normal metabolism and to compare this with the fate of folate under altered conditions or physiological states. Additionally, the availability of stable isotopes of folate affords the luxury of evaluating several forms of folate simultaneously and allows the researcher to determine the kinetics of absorption and turnover of the administered compound (Gregory and Toth, 1990). Other developments that have facilitated research in this area include the development of improved methods for separating various folate monoglutamates using high-performance liquid chromatography (HPLC) (Kashani and Cooper, 1985; Wilson and Horne, 1984); the development of affinity chromatography columns that allow for easier separation of folates from the sample (Selhub et al., 1980), which ultimately results in cleaner chromatographic separation using HPLC (Selhub, 1989); and the development of

a mass spectrometric method to quantify the ratio of unlabeled versus labeled folates (Toth and Gregory, 1988).

The first published studies of an in vivo application of stable-isotopically labeled folates in human subjects were performed by Gregory and coworkers (Gregory and Toth, 1988a; Gregory et al., 1990). The initial report (Gregory and Toth, 1988a) described the results of a preliminary investigation of gas chromatography/mass spectral (GCMS) analysis of urinary folates. The second study (Gregory et al., 1990) evaluated the adequacy of a saturation regimen of 2 mg/d of folic acid and the effectiveness of simultaneous administration of two forms of deuterium-labeled folate. Subjects participating in this study received 2 mg/d unlabeled folic acid for one week to enhance urinary excretion of absorbed folates. On the morning prior to administration of the deuterium-labeled folates, the subjects collected a 24 hour urine sample. The next morning, after an overnight fast, subjects consumed apple juice containing two different deuterium-labeled folates. The subjects collected their urine for 48 hours following the treatment. A constant diet was consumed beginning at the time of the pre-dose urine collection through the end of the post-dose urine collection. Dietary and total urinary folate concentration was determined by microbiological assay using *L. casei*. Urine samples were also prepared for mass spectral analysis of labeled folates, after which the molar ratio of each species of folate in the sample was calculated. The



results of these studies confirmed that stable-isotopically labeled folates are well-suited for in vivo studies and that the protocol outlined above is suitable for studying many aspects of folate bioavailability and in vivo kinetics (Gregory et al., 1990). Recently, Von der Porten et al. (1992) used this methodology to study in vivo folate kinetics in human subjects supplemented with deuterium-labeled folic acid for four weeks. By using deuterium-labeled folate, these researchers were able to determine rate constants and isotope enrichment, which subsequently enabled them to assess tissue uptake and equilibration, in vivo turnover rates and body pool sizes of folate. It is expected that further application of this technique will greatly enhance our knowledge of folate metabolism and improve our ability to make appropriate nutritional recommendations.

## Zinc

### Chemistry

Zinc has an atomic weight of 65.37. It is a first series transition element with the electronic configuration [Argon]  $4s^2 3d^{10}$ . This configuration confers properties to zinc that distinguish it from other transition metals. For example, zinc is diamagnetic rather than paramagnetic, and although it can exist in several valence states, it is resistant to oxidation and is found almost universally as the divalent ion ( $Zn^{2+}$ ). Another property of zinc that is relevant to its

specific biological functions is its ability to form stable complexes with side chains of proteins. The high charge density of  $\text{Zn}^{2+}$  allows this metal to function as a Lewis acid to withdraw electrons from electron-rich functional groups of ligands resulting in the formation of noncovalently bound coordination complexes. These features make zinc ideally suited for its involvement in enzyme function and structure (Solomons, 1988; Williams, 1989).

### Absorption

The process of zinc absorption includes the uptake of zinc by the intestinal mucosal cells, movement of zinc through the mucosal cells and transfer into the portal circulation. Paracellular movement of zinc to the portal circulation may also occur. These aspects of zinc absorption are discussed in the following paragraphs.

Although the study of zinc absorption has been given much attention, the precise mechanism, location and control of this process have not been fully delineated (Cousins and Hempe, 1990). Differences in experimental conditions may account for the conflicting results of in vivo and in vitro experiments designed to study zinc absorption, since a multitude of host and environmental factors have been shown to influence this process (Solomons and Cousins, 1984).

Zinc is absorbed throughout the small intestine, but the segment with the highest capacity to absorb zinc has not yet

been determined (Cousins and Hempe, 1990). Rat studies used to identify the major absorptive site have produced variable results, with some studies (Davies, 1980; Methfessel and Spencer, 1973; Van Campen and Mitchell, 1965) suggesting that the greatest amount of zinc uptake occurs in the duodenum, and others (Antonson et al., 1979; Emes and Arthur, 1975) suggesting that greater zinc uptake occurs in the distal portions of the intestine. Although Sahagian et al. (1966) found the concentration of zinc from normal rat intestine to be fairly uniform throughout each region, zinc uptake by strips of rat intestine was greater in the duodenal and ileal segments compared to the jejunal segment. These findings are in contrast to those of an in vivo intestinal perfusion study (Matseshe et al., 1980) conducted in humans. In this study, Matseshe et al. found that more zinc left the distal duodenum than what was contained in a test meal, suggesting that endogenous secretions contributed to the total intraluminal zinc concentration. For this reason, net duodenal zinc disappearance, if any, could not be determined. Once the intraluminal contents passed into the jejunum, zinc disappeared gradually but incompletely. Ileal zinc absorption was not determined in this study. In fact, all portions of the small intestine may be functionally important in terms of zinc absorption, with the duodenum having first access to dietary and endogenous zinc, and subsequent sections having the benefit of the action of digestive processes that may

increase the accessibility of this nutrient (Cousins and Hempe, 1990; Lönnerdal, 1989a).

The first phase of the absorptive process involves the movement of zinc from the intestinal lumen into the mucosal cell. Evidence that zinc absorption occurs rapidly and involves a saturable, carrier-mediated component, as well as nonsaturable diffusion has come from several rat studies. Davies (1980) exposed ligated duodenal loops from rats to different concentrations of zinc and found that these segments exhibited saturation kinetics at low luminal zinc concentrations. At higher luminal zinc concentrations, zinc uptake was linear suggesting that zinc absorption also occurred by diffusion. Menard and Cousins (1983) reported similar results using isolated brush border membrane vesicles from rats. Further evidence for a mediated component of zinc absorption was derived from experiments with rats previously fed a zinc-adequate or a zinc-deficient diet (Steel and Cousins, 1985). Zinc absorption, as determined from zinc accumulation in the portal perfusate, revealed that absorption was saturable in both groups, and that zinc-deficient rats had a more rapid rate of zinc absorption at all luminal zinc concentrations. Zinc absorption appeared to involve both mediated and nonmediated components, with most of the absorption occurring by means of the mediated component in the zinc-deficient group. These results suggest that the saturable process is stimulated by zinc depletion, whereas the

nonsaturable process is unaffected by zinc deficiency and proceeds in proportion to the intraluminal zinc concentration. A more recent study conducted by Hoadley et al. (1987) is in agreement with the findings of previous studies suggesting that zinc absorption involves two kinetic processes.

Although there is general agreement that zinc uptake by the intestinal mucosal cell involves a saturable, carrier-mediated component, as well as nonsaturable diffusion, it is not known if these processes represent components of a single transport step occurring at the brush border membrane, or if movement from the lumen to the portal circulation occurs by two independent routes: transcellular and paracellular. (Cousins and Hempe, 1990). Transcellular movement may involve a protein that has recently been identified (Hempe and Cousins, 1991), purified and partially characterized (Khoo and Cousins, 1993). This protein, known as cysteine-rich intestinal protein is a low molecular weight, zinc-binding, cytosolic protein isolated from intestinal mucosal cells. Cysteine-rich intestinal protein has two possible zinc binding sites, and it is hypothesized that this protein serves as an intracellular zinc carrier. This protein appears to bind more zinc when intestinal metallothionein is not induced, suggesting that intestinal metallothionein may interact with cysteine-rich intestinal protein to regulate zinc absorption and transport (Khoo and Cousins, 1993).

Movement of zinc from the intestinal lumen into the mucosal cell may be affected by the intraluminal environment. Zinc liberated from food matrices during the digestive process may remain as the free ion or may form coordination complexes with endogenous (pancreatic, biliary or mucosal) or exogenous (dietary) intraluminal ligands (Solomons and Cousins, 1984). Studies investigating the effect of intraluminal binding ligands on zinc uptake have suggested that ligand binding may affect zinc absorption by altering intestinal membrane permeability and/or zinc gradients across intestinal cell membranes (Cousins, 1985; Solomons and Cousins, 1984). However, while there is little question that zinc-binding ligands affect zinc absorption, it is doubtful that this process is a prerequisite for movement of zinc across the membrane surface (Cousins and Hempe, 1990).

Once zinc is inside the intestinal mucosal cell, it binds to a variety of high molecular weight ligands, as well as to intestinal metallothionein, a low molecular weight, cysteine-rich metalloprotein. The amount of zinc bound to intestinal metallothionein varies according to zinc status and intake. Zinc binding to intestinal metallothionein restricts the movement of zinc from the cell, thereby contributing to the regulation of zinc absorption. Intestinal metallothionein synthesis is induced at the transcriptional level by high dietary zinc (Blalock et al., 1988; Menard et al., 1981). When the rate of intestinal metallothionein synthesis is high, net

zinc release to the portal circulation is curtailed (Menard et al., 1981). In zinc deficiency, intestinal metallothionein concentration is low and more zinc is released into the portal circulation (Hoadley, et al., 1988). Thus, it appears that the induction of metallothionein synthesis in intestinal mucosal cells could provide for the efficient regulation of zinc transfer to the vascular compartment.

Based on the results of rat studies (Davies, 1980; Smith and Cousins, 1980), transfer of zinc from the mucosal cell to the portal circulation occurs more slowly than uptake and accumulation within the cell. According to Davies (1980), transfer of zinc into the portal circulation may occur in two stages: 1) rapid transfer occurring over the first 30 minutes; and 2) slower transfer occurring from 30 minutes to six hours after receiving the dose, which may represent the release of intracellularly bound zinc from zinc-binding proteins. Consequently, movement of zinc from the intestinal mucosal cell into the vascular compartment may be the rate-limiting step in zinc absorption (Smith and Cousins, 1980). At low luminal zinc concentrations, much of the zinc available is released into the portal circulation; however, as the luminal zinc concentration increases, less of this nutrient is transported to the vascular system (Hambidge et al., 1986; Smith and Cousins, 1980). An exception to this phenomenon occurs when excessive loads of luminal zinc are available. In

this case, the ability to control the release of zinc into the circulation is diminished (Smith and Cousins, 1980).

### Transport, Distribution and Metabolism

Although it had been proposed that transferrin was responsible for portal zinc transport (Evans and Winter, 1975), several lines of investigation have shown that albumin is the principal portal transport protein for zinc (Smith et al., 1979). Smith et al. (1979) demonstrated the importance of albumin in portal transport by comparing the percent of  $^{65}\text{Zn}$  absorbed when the vascular perfusate composition was varied. The absence of albumin from the perfusate resulted in negligible zinc transfer to the portal circulation, whereas the isosmotic replacement of all plasma proteins with albumin resulted in a twofold increase in zinc transfer over that of the control perfusate. These data intimate that the extent of zinc absorption may be affected by the concentration of albumin in the blood (Cousins, 1985). Consequently, zinc absorption may be impaired in disease states associated with the development of hypoalbuminemia.

Zinc entering the portal circulation is rapidly transported to the liver. The liver is the primary organ involved in zinc metabolism, and a large portion of the zinc in portal blood is exchanged with the liver (Cousins and Hempe, 1990). In vitro experiments with rat liver parenchymal cells demonstrated that zinc uptake was temperature and



energy-dependent, followed saturation kinetics and occurred in two phases (Failla and Cousins, 1978a). The first phase was characterized by rapid, saturable uptake. This was followed by a slower phase comprised of both saturable and linear components. Maximal uptake occurred at the normal plasma zinc concentration (Cousins, 1989; Failla and Cousins, 1978a). Zinc accumulation by these cells increased when physiological concentrations of certain adrenal corticosteroids were added suggesting that these hormones may perform an essential role in the regulation of hepatic zinc metabolism. As a corollary, it is possible that zinc has a role in glucocorticoid-mediated alterations of hepatic metabolic processes (Failla and Cousins, 1978b).

Distribution of zinc to the extrahepatic tissues occurs mainly via the plasma. The plasma contains approximately 10 to 20% of the zinc in whole blood, whereas the erythron and leukocytes contain the major portion of zinc in the blood. Most of the plasma zinc is bound to albumin, although alpha-2-macroglobulin, transferrin, histidine and cysteine may also transport small amounts of this metal. Zinc bound to albumin is considered to be loosely bound and represents the metabolically active, exchangeable zinc pool in the blood. This pool is responsive to acute and chronic changes related to stress, infection and dietary zinc. In contrast to plasma zinc, the zinc in erythrocytes is mostly associated with carbonic anhydrase, although small amounts may be associated

with superoxide dismutase and metallothionein (Cousins, 1989; Cousins and Hempe, 1990; DiSilvestro and Cousins, 1983).

The rate of zinc incorporation into extrahepatic tissues varies, as does the rate of zinc turnover in these tissues. Zinc accumulation and turnover occurs rapidly in the kidney, pancreas and spleen (Hambidge, et al., 1986). The rate of zinc uptake by skeletal muscle and the central nervous system is relatively slow, and the zinc in these tissues remains tightly bound for long periods of time (Hambidge et al., 1986). The zinc incorporated into hair is not available for exchange either. Tissue-specific redistribution of body zinc can occur during periods of zinc deprivation, stress and infection/inflammation (Cousins and Leinart, 1988; Dunn and Cousins, 1989; Giugliano and Millward, 1984; Huber and Cousins, 1988; Jackson et al., 1982). For example, the zinc concentration of muscle in zinc-deficient rats is protected, while the concentration in bone, liver and plasma declines (Giugliano and Millward, 1984; Jackson et al., 1982). This redistribution may occur as a result of tissue-specific induction of metallothionein.

In general, the largest concentration of intracellular zinc is found in the cytosol, although smaller amounts are present in the nuclear, microsomal and mitochondrial fractions of cells (Hambidge et al., 1986). Zinc is also present in the membrane and may enhance membrane stability (Bettger and O'Dell, 1981). Within the cytosol, zinc is primarily bound to

large molecular weight proteins. These proteins may be zinc metalloenzymes. In contrast, the amount of zinc bound to metallothionein is relatively low under normal dietary conditions; however, when dietary zinc is increased, the metallothionein gene is induced and metallothionein synthesis is elevated (Cousins and Lee-Ambrose, 1992; Cousins and Leinart, 1988; Huber and Cousins, 1988). Consequently, increased zinc binding to metallothionein in the liver, pancreas, kidney and muscle occurs. Zinc may regulate the expression of the metallothionein gene through a specific nuclear metal-binding protein that also binds to unique DNA sequences in the promoter region (Cousins and Hempe, 1990). Synthesis of metallothionein, particularly in the liver, may also occur in response to hormones, as well as physiological stimuli such as stress, acute infection and shock (Cousins and Hempe, 1990; Hambidge et al., 1986).

### Excretion

The major route of zinc excretion is via the feces. Zinc in the feces is derived from unabsorbed dietary zinc, as well as endogenously secreted zinc. Sources of endogenous zinc include pancreatic, biliary and mucosal secretions, as well as zinc present in desquamated mucosal cells. Usually, endogenously secreted zinc is efficiently reabsorbed, but intraluminal factors such as the presence of phytic acid may

decrease the efficiency of absorption (Cousins and Hempe, 1990). Additionally, dietary intake of zinc may affect reabsorption, with more zinc of endogenous origin being excreted in the feces at higher levels of zinc intake (Jackson et al., 1984).

Urinary zinc losses account for a fairly small amount of the zinc excreted under normal physiologic conditions. The usual range of zinc excreted in the urine of healthy adults is about 300 to 600  $\mu\text{g/d}$  (Gibson, 1990). Urinary zinc excretion has been shown to decrease in human subjects consuming a zinc-deficient experimental diet (0.28 mg zinc/d) (Baer and King, 1984). Injury, burns, infection, acute starvation and pathologic conditions resulting in excessive muscle catabolism have been associated with clinically significant increases in urinary zinc excretion. Hyperzincuria is known to occur in conjunction with proteinuria due to kidney dysfunction and in patients with sickle cell disease and cirrhosis of the liver (Cousins and Hempe, 1990; Gibson, 1990).

Dermal losses due to sweating and to the sloughing of epithelial tissue also account for some of the zinc lost from the body. As reported in a review by Hambidge et al. (1986), the total amount of zinc lost in sweat from adults living in a temperate North American climate has been estimated to be 0.4 to 2.8 mg/d. Dermal zinc losses have been found to be influenced by dietary zinc intake, with reduced losses occurring when zinc intake is marginal, and losses increasing

following zinc repletion (Milne et al., 1984). This apparent conservation of zinc during a period of inadequate zinc intake may represent a homeostatic mechanism (Hambidge et al., 1986).

Menstrual and seminal excretions represent additional routes by which zinc losses may occur in females and males, respectively. The average amount of zinc lost with each menstrual cycle represents a loss of approximately 15  $\mu\text{g/d}$  over the course of a month (Hambidge et al., 1986). Seminal zinc losses are estimated to be approximately 0.6 mg/ejaculum (Baer and King, 1984).

### Zinc Homeostasis

Whole body zinc homeostasis is achieved primarily by changes in absorption and excretion in response to changes in dietary zinc intake. The results of an animal study in which rats were fed increasing amounts of zinc (Coppen and Davies, 1987) suggest that zinc homeostasis at lower levels of zinc intake is a function of increased efficiency of absorption and retention, but when dietary zinc intake is excessive, homeostasis is modulated solely by changes in zinc excretion.

### Biochemical Functions

The unique chemistry of zinc and its biological abundance make it ideally suited for a multitude of biochemical and physiological functions. Of these functions, zinc is most noted for its role as a component of over 60 different

metalloenzymes, including those involved in the metabolism of proteins, carbohydrates, lipids and nucleoproteins. The specific metalloenzyme roles of zinc have been characterized as catalytic, structural and regulatory. The catalytic action of zinc is probably due to its ability to bind directly with the substrate and/or through a metal-bound molecule of water. Examples of zinc metalloenzymes with catalytic roles include carbonic anhydrase and carboxypeptidase. The structural role of zinc is related to its ability to provide structural integrity to zinc-requiring enzymes, such as superoxide dismutase, by stabilizing the quaternary structure of the enzyme complex. Alteration of this structure due to the loss of zinc results in disruption of enzyme activity. Apart from its catalytic and structural roles, zinc may also serve a regulatory function by inhibiting enzymatic function, as it does in fructose-1,6-bisphosphatase (Hambidge et al., 1986).

There is evidence that in addition to the enzymatic functions of zinc, this nutrient may have important nonenzymatic biological functions. For example, zinc may play a role in gene transcription and expression by promoting structural changes in DNA-binding proteins that allow these proteins to bind more strongly or specifically to DNA via motifs called zinc fingers. Although the impact of zinc deficiency on zinc fingers requires further investigation, it is possible that zinc deficiency could result in improper folding or structural configuration of the DNA-binding

proteins, resulting in altered gene expression, cell dysfunction and ultimately, overt clinical manifestations of zinc deficiency (Cousins and Hempe, 1990).

Bettger and O'Dell (1981) have suggested that zinc may function to stabilize cell membrane structure. These researchers found that erythrocytes from zinc-deficient rats contained significantly less zinc and exhibited increased osmotic fragility compared to erythrocytes from control animals. The fact that elevated lipid peroxidation has been observed in tissues from zinc-deficient animals (Sullivan et al., 1980) lends support to this idea and may provide an explanation for the development of skin disorders and other symptoms associated with zinc deficiency. Although a mechanism for this proposed cytoprotective function has not been identified, it is possible that stabilization may occur through phospholipid or thiol group linkages with zinc (Cousins and Hempe, 1990).

The numerous physiologic functions ascribed to zinc are listed in Table 2-2. Most of these functions have been identified based on the results of animal and/or human studies in which a zinc-deficient state was experimentally induced, or clinical observations of zinc-deficient humans. In many cases, the underlying biochemical mechanism(s) responsible for the physiological effect has not been determined.

Table 2-2. Physiologic functions of zinc.

Antioxidant role via metalloproteins
Cellular growth
Cellular replication
Fertility and reproduction
Hormone production, storage and secretion
Immune system development and maintenance
Protein, carbohydrate and lipid metabolism
Sexual maturation
Taste and appetite

### Recommended Dietary Allowances for Zinc

Estimates of human zinc requirements have been made using balance studies and by measuring endogenous zinc losses along with fractional zinc absorption. Neither of these methods is entirely satisfactory because the results can be influenced by homeostatic mechanisms that control zinc absorption and excretion. An alternative approach would be to determine the amount of zinc that maintains normal physiological and metabolic functions. This approach has been limited by the lack of a sensitive and specific indicator(s) of zinc status (King, 1986).

The 1989 Recommended Dietary Allowances (RDA) for zinc were based on the results of balance studies and estimated zinc losses in healthy adults. Balance studies have suggested



that at least 12 mg zinc/d is necessary to achieve equilibrium. Estimates of zinc losses range from 2.2 to 2.8 mg/d in healthy young males consuming a mixed diet. Based on these loss estimates, it has been assumed that an average of approximately 2.5 mg/d absorbed zinc is needed to maintain equilibrium. Using an absorption efficiency of 20% to allow for poor zinc absorption associated with high fiber diets, the resulting dietary requirement amounts to 12.5 mg/d, which closely approximates the estimate determined from balance studies. To allow for a margin of safety, the 1989 RDA for zinc has been set at 15 mg/d for adolescent and adult men, whereas the RDA for adolescent and adult females has been set at 12 mg/d because of their smaller body size (Food and Nutrition Board, 1989b). The RDA for zinc for elderly Americans is set at the same level as that for younger adults, although the results of a stable isotope study (Turnland et al., 1986) suggest the possibility that this population group may have a higher requirement due to a reduced ability to absorb zinc. Conversely, the reduction in zinc absorption may reflect a lower requirement for absorbed zinc.

The increase in the requirement for zinc during pregnancy may be a reflection of the important role zinc plays in cellular replication and differentiation. Estimates of the average amount of additional zinc needed during pregnancy (i.e. approximately 100 mg zinc/normal pregnancy) have been made by Sandstead (1973) and by Swanson and King (1987).

Based on these estimates and the lack of evidence for increased absorption efficiency during pregnancy (Swanson et al., 1983), a dietary intake of 15 mg/d is recommended during pregnancy (Food and Nutrition Board, 1989b).

The average daily milk production and the average zinc content of human milk during the first and second six months after delivery were used to estimate the daily requirement during lactation. This estimate was adjusted to account for an absorption efficiency of 20% and a coefficient of variation of 12.5%, resulting in a recommended allowance of 19 mg/d and 16 mg/d during the first and second six months of lactation, respectively (Food and Nutrition Board, 1989b).

Full-term, breast-fed infants rarely show signs of zinc deficiency, so it has been assumed that the zinc content of human milk, along with the infant's liver stores, are adequate to meet daily requirements during the first six months of life (Food and Nutrition Board, 1989b). The bioavailability of zinc from human milk is higher than that from infant formulas (Blakeborough et al., 1986; Sandström et al., 1983). The high concentration of zinc-binding ligands such as citric acid and/or picolinic acid was once thought to be responsible for the higher bioavailability (Eckhert et al., 1977). However, the high lactalbumin content of human milk, as opposed to the high casein content of cow's milk-based formulas, appears to be the major determinant of zinc bioavailability (Cousins and Smith 1980; Lönnerdal, 1989b; Roth and Kirchgessner, 1985;

Sandström et al., 1983). Although the zinc content of human milk decreases as the infant ages, the contribution of zinc from solid foods is thought to be sufficient to meet the infant's needs during the second six months of life.

Compared to breast-fed infants, the estimated requirement for formula-fed infants is higher because the bioavailability of zinc from formula, particularly soy-based formulas, is lower (Casey et al., 1981; Lönnerdal et al., 1984). The current RDA for formula-fed infants was based on a study (Walravens and Hambidge, 1976) demonstrating better growth of male infants fed a formula supplemented with 4 mg zinc/L (i.e. 5.8 mg zinc/L) compared with infants consuming the same formula without additional zinc (i.e. 1.8 mg zinc/L). Assuming that formula consumption is 750 mL/d, plus two standard deviations, the RDA for formula-fed infants was set at 5 mg/d (Food and Nutrition Board, 1989).

Consideration has been given to the possibility that iron-fortified infant formulas or infant foods may impair zinc absorption and adversely affect zinc status (Craig et al., 1984; Fairweather-Tait and Southon, 1989). However, the American Academy of Pediatrics Committee on Nutrition (1989) has rendered the opinion that the available evidence does not support a detrimental effect of iron-fortified formulas on infants' zinc status.

The recommended dietary allowance for zinc for preadolescent children is 10 mg/d. This recommendation was

made based on a study of Spanish-American children (Walravens et al., 1983) with zinc intakes of approximately 5 to 6 mg/d. These children displayed signs of marginal zinc deficiency and their height-for-age was below the tenth percentile. When their diet was supplemented to a total intake of 10 mg/d, their rate of linear growth improved. Evidence of a growth-limiting effect due to marginal zinc deficiency, and a growth-enhancing effect due to zinc supplementation, has also been demonstrated in Southern Ontario boys aged 5 to 7 (Gibson et al., 1989).

#### Effects of High Doses of Zinc

Cases of acute and chronic zinc toxicity have been reported in humans. Accounts of acute toxicity are relatively rare and have been primarily associated with food poisoning incidents (Fosmire, 1990). In these cases (Brown et al., 1964), acidic foods or beverages were stored in galvanized containers for long periods of time. Presumably, sufficient zinc was leached from the coating of these containers to cause toxic manifestations which included nausea, vomiting, epigastric pain, abdominal cramps and diarrhea. Different symptoms, including lethargy, light-headedness and slight staggering of gait, were reported (Murphy, 1970) in a case where 12 g elemental zinc was ingested over a period of 36 h. Fosmire (1990) has suggested that the form of zinc salt

ingested may influence which manifestations of toxicity will be observed.

Zinc toxicity resulting from moderately elevated intakes of zinc (i.e. 100 to 300 mg/d) is more common than acute zinc toxicity (Fosmire, 1990). Potential consequences of prolonged intakes within this range include induction of copper deficiency, impaired immune response and altered plasma lipoprotein profiles.

Copper deficiency, manifested by low plasma copper, anemia and neutropenia has been reported in patients with sickle cell anemia (Prasad et al., 1978b) and patients with nonresponsive celiac disease (Porter et al., 1977) treated with 150 mg zinc/d for 23 or 13 months, respectively. Impaired copper status has been reported with lower levels of supplementation as well (i.e. 50 mg/d for 6 or 10 weeks) (Fischer et al., 1984; Yadrick et al., 1989).

Evidence of impaired immune response in young men consuming 300 mg zinc/d for 6 weeks was reported by Chandra, 1984; however, supplementation with 100 mg zinc/d for 3 months in an elderly population did not result in impaired immune status (Bogden et al., 1988). Differences in the ages of the subjects and the supplementation regimens may account for the discrepant results (Fosmire, 1990).

Altered lipoprotein profiles have been reported (Chandra, 1984) in subjects given 300 mg zinc/d for 6 weeks. This dose of zinc resulted in a significant decrease in high-density

lipoprotein cholesterol and a significant increase in low-density lipoprotein cholesterol levels. Reduced high-density lipoprotein cholesterol levels have also been reported in subjects consuming 160 mg zinc/d for 6 weeks (Hooper et al., 1980).

#### Food Sources of Zinc, Usual Intakes and Bioavailability

Major dietary sources of zinc include foods with a high protein content such as oysters and other shellfish, meat, liver and dairy products. The zinc content of meat varies depending on the species and the specific tissue, with dark red meat generally containing more zinc than white meat. Whole grains, legumes and nuts are also good sources of this nutrient, but the zinc in these foods is generally less available for absorption and can be affected by growing conditions and processing. Vegetables and fruits contain small amounts of zinc. Sweets, fats and oils are relatively low in zinc.

The zinc content of the United States food supply in 1985 was estimated to be 12.3 mg/person/d (Moser-Veillon, 1990). Although this estimate does not meet the zinc RDA for adult men, it should be recognized that the per-person data included women and children for whom the zinc RDA is lower. This estimate assumes an equal distribution of foods and nutrients across the entire population and does not account for the effect of food choices or zinc bioavailability.

Data from the Food and Drug Administration's (FDA) Total Diet Study (Pennington and Young, 1991) indicate that American infants generally meet their RDA for zinc. In the case of young children, the findings of FDA's Total Diet Study and the Nationwide Food Consumption Survey (United States Department of Agriculture, 1987), are not as encouraging, with many children consuming diets with zinc contents below the RDA. During adolescence and adulthood, gender differences in zinc intakes become apparent, with males tending to have more adequate intakes of this nutrient. For example, FDA's Total Diet Study found that the diets of teenage boys, but not girls, met the RDA for zinc. Similarly, the 1985 Nationwide Food Consumption Survey (United States Department of Agriculture, 1986) found that men between the ages of 19 to 50 years consumed an average of 94% of their RDA for zinc, while FDA's Total Diet Study reported an average of 109% of the RDA for men between the ages of 25 to 30 years. In contrast, the 1986 Nationwide Food Consumption Survey (United States Department of Agriculture, 1987) found that women between the ages of 20 to 49 years consumed about half of the RDA for zinc. Low dietary zinc intakes among women have been confirmed by other studies including FDA's Total Diet Study and the National Health and Nutrition Examination Survey II. The difference in total zinc intake between males and females has been attributed to differences in energy consumption since

the zinc density of diets consumed by men and women were similar (Moser-Veillon, 1990).

Since zinc intake is associated with caloric consumption, it is not surprising that elderly people, who may restrict their food intake due to illness, poverty and/or difficulty in chewing, often have lower zinc intakes than younger adults. The estimated zinc intake of elderly people is 7 to 10 mg/d (Greger, 1989). Zinc bioavailability may also be reduced in this group due to reduced meat consumption and increased consumption of phytic acid containing foods such as cereals.

As discussed in a review by Cousins and Hempe (1990), even though the zinc content of foods can be used to estimate dietary intake of this nutrient, the bioavailability of zinc from different foods and food combinations is highly variable. For this reason, dietary intake may be a poor indicator of the amount of zinc that is actually available for use by the body. A more appropriate indicator of zinc supply would be the amount of bioavailable zinc in the diet; however, this is difficult to determine because estimates can be affected by zinc status, nutrient interactions and the experimental methods used to make these determinations (Cousins and Hempe, 1990).

Factors that may enhance or inhibit zinc availability and absorption have been reviewed by Cousins and Hempe (1990). Substances that may form insoluble complexes with zinc and thus reduce the availability of this nutrient include: fiber,



phytic acid, oxalic acid, calcium, iron and high concentrations of ethylenediamine tetraacetic acid (EDTA). Dietary factors that may enhance zinc absorption, such as amino acids (histidine, cysteine, lysine and glycine), citric acid, picolinic acid and low concentrations of EDTA, are thought to improve zinc absorption by forming digestible and absorbable zinc chelates.

Rat studies have shown that phytic acid has an adverse effect on zinc absorption when the phytic acid:zinc ratio exceeds 12 to 15 (Morris and Ellis, 1980). Phytic acid alone, however, does not appear to adversely effect zinc bioavailability in humans at a phytic acid:zinc ratio of 10 (Ellis et al., 1987). Estimates (Ellis et al., 1982) of the phytic acid:zinc ratio of omnivorous and vegetarian diets typically consumed in the United States (i.e. a molar ratio of 3.3 for meat-based diets, 4.5 for lacto-ovo vegetarian diets and 7.6 for soy-based diets) suggest that the phytic acid content is not high enough to impair zinc bioavailability. The effect of phytic acid is accentuated, however, in the presence of high intraluminal calcium (Forbes and Erdman, 1983). Based on human experiments (Ellis et al., 1987) applying the phytic acid x calcium:zinc ratio, phytic acid does not appear to adversely effect zinc absorption from omnivorous diets. It is possible, however, that zinc bioavailability may be impaired in lacto-ovo vegetarians consuming large quantities of calcium.

A competitive interaction between iron and zinc may also exist; however, the results of human studies are controversial. A possible explanation for the conflicting results may be due to factors such as the form of iron, the presence of a meal and/or the amounts of zinc and iron fed (Storey and Greger, 1987). For example, when equal amounts of ferrous iron and zinc (as sulfate) were ingested simultaneously, zinc absorption was depressed; however, zinc absorption was not affected when heme iron or a food source of zinc were used (Solomons and Jacob, 1981). Additionally, when human subjects consumed iron supplements with a meal, zinc absorption was not impaired (Sandström et al., 1985). It is likely that under most dietary conditions, an interaction between iron and zinc is not sufficient to influence zinc requirements; however, zinc absorption may be adversely affected in pregnant women taking iron supplements (Simmer et al., 1987).

Animal studies (Stuart et al., 1986) have shown that zinc absorption increases when dietary protein increases, and that zinc is more bioavailable from animal than plant protein sources. The effect of protein on zinc absorption in humans, however, is controversial. The inconsistent effect of protein on zinc absorption in human studies may be due to other factors associated with proteins such as phosphorus and phytic acid content, as well as zinc status and previous intake. As noted above, some amino acids have been associated with

improved zinc absorption because they form soluble complexes with zinc (Solomons and Cousins, 1984). It is possible that part of the positive effect of protein on zinc absorption observed in animal studies is due to the effect of additional amounts of these amino acids.

### Factors Affecting Zinc Status

As noted above, the physiological stage of growth and development affects zinc requirements, and the amount and bioavailability of dietary zinc consumed, relative to zinc need, has an impact on zinc status. Other factors that can affect zinc status include diseases, alcohol consumption and medication use. The potential effect of these factors on zinc status is discussed below.

Diseases. Zinc intake, absorption, excretion and/or requirements can be affected by the presence of certain diseases (Cunnane, 1988), and many diseases are associated with anorexia and/or decreased taste acuity. These problems may reduce overall food intake, resulting in a reduction in zinc intake. Impaired zinc absorption resulting from diseases associated with intestinal mucosal damage, such as Crohn's disease (McClain et al., 1980; Solomons et al., 1977), and from diseases in which there is a lack of appropriate absorption ligands, such as acrodermatitis enteropathica (Aggett, 1989) and cystic fibrosis (Caillie-Bertrand et al., 1982), can also adversely affect zinc status. Impaired zinc

status has also been reported in diseases associated with excessive zinc losses such as sickle cell disease (Prasad et al., 1976) and insulin-dependent diabetes mellitus (Canfield et al., 1984). Increased zinc requirements are associated with neoplastic diseases due to rapid tissue turnover (Solomons, 1988).

Alcohol. The etiology of impaired zinc status observed in some alcoholics is probably multifactorial, with inadequate dietary intake, intestinal malabsorption, pancreatic insufficiency, decreased albumin affinity and excessive urinary excretion contributing to abnormal zinc metabolism (Solomons, 1988). Zinc status does not appear to be compromised as a result of occasional alcohol use, or in all individuals suffering from alcoholism, suggesting that the effect on overall zinc homeostasis is dependent on the amount and frequency of alcohol consumed and the extent of alcohol-induced tissue damage (Cunanne, 1988).

Medications. Medications that may interfere with zinc status include antineoplastic agents, aspirin and penicillamine. Antineoplastic agents and aspirin may chelate zinc, making it less available to the body. Penicillamine interferes with zinc status by increasing urinary zinc excretion (Cunanne, 1988).

### Assessment of Zinc Status

Assessment of zinc status has been difficult because a specific and sensitive indicator has not been available for this purpose. In the absence of a single reliable measure of zinc status, a variety of biochemical and functional indices have been used. These indices include measurements of zinc in tissues or fluids such as plasma/serum, erythrocytes, leukocytes, neutrophils, urine and hair. Functional indices such as changes in zinc metalloenzyme activity, dark adaptation, taste acuity and macrophage chemotaxis have also been used to assess zinc status. The limitations of these indices are discussed in the following paragraphs.

Plasma/serum zinc concentration. Plasma or serum zinc concentration is the most commonly used index of zinc status (Cousins and Hempe, 1990; Solomons, 1979). Individuals with fasting morning plasma/serum zinc values below 70  $\mu\text{g/dL}$  ( $< 10.71 \mu\text{mol/L}$ ) are considered to be at risk for zinc deficiency (Gibson, 1990). Decreased plasma/serum zinc concentrations have been reported in patients whose sole source of nutrition consisted of total parenteral nutrition solutions that did not contain zinc (Arakawa et al., 1976; Fleming et al., 1976). Experimentally-induced severe zinc deficiency has also resulted in decreased plasma/serum concentrations of this nutrient (Baer and King, 1984; Gordon et al., 1982; Hess et al., 1977; Prasad et al., 1978a).

Studies of pregnant women suggest that the normal pattern of decline in plasma zinc throughout gestation may be useful as an indicator of zinc utilization, and that deviations from this pattern may indicate abnormal utilization, and thus, impaired status (Swanson and King, 1987).

One of the problems associated with the use of plasma/serum zinc concentration as an index of zinc status is that this measure is subject to homeostatic control. As a result, normal plasma/serum zinc concentrations may be maintained despite inadequate zinc intake and/or absorption. For example, in a study designed to evaluate zinc utilization in young men fed adequate and low zinc intakes, Wada et al. (1985) found that plasma zinc concentrations did not fall when dietary zinc was decreased to 5.5 mg/d; however, fecal zinc excretion was reduced. Similarly, zinc losses in sweat decreased by 65%, but plasma zinc concentrations did not decline during an 18 week study of subjects consuming 3.6 mg zinc/d (Milne et al., 1983). Studies of infants and children suggest that mild zinc deficiency is associated with growth retardation in the absence of changes in plasma zinc concentrations (Walravens and Hambidge, 1976; Walravens et al., 1983; and Walravens et al., 1989). These findings suggest that the initial response to mild zinc deficiency is a reduction in growth rate or zinc excretion. As discussed by King (1990), these adaptive responses appear to result in the maintenance of plasma/serum zinc concentrations, and it is not

until the homeostatic capacity is exceeded that plasma/serum zinc concentrations decline. The eventual reduction in plasma/serum zinc represents a loss of zinc from bone and liver and signals the impending development of metabolic and clinical signs of zinc deficiency (King, 1990). Thus, plasma/serum zinc concentrations is a marker of the size of the exchangeable zinc pool (King, 1990).

Another problem associated with the use of plasma/serum zinc concentration as an index of zinc status is that it is altered by several nonnutritional factors. These factors include: infection (Solomons et al., 1978; Wannemacher et al., 1975), diurnal variations (Gordon et al., 1982), hormonal state (Cousins, 1985) and short-term fasting (Henry and Elmes, 1975). Changes in circulating zinc associated with these factors are not due to altered zinc status, but to metabolic disturbances that result in zinc redistribution to other tissues (Golden, 1989). For example,  $^{65}\text{Zn}$  uptake was increased in the liver, bone marrow and thymus and reduced in the bone, skin and intestine when interleukin-1 was administered to rats (Cousins and Leinart, 1988). This redistribution of tissue zinc was accompanied by a temporary decline in circulating zinc. Consequently, plasma/serum zinc concentration can not be used as a specific indicator of zinc status unless the effects of other metabolic conditions can be differentiated from the effects due to actual changes in zinc status (Golden, 1989; King, 1990).

Oral zinc tolerance test. This test measures the increase in plasma zinc from baseline (i.e. fasting) to four hours after the ingestion of a pharmacological dose of zinc. The results of the oral zinc tolerance test compare favorably with those obtained by direct measurements of  $^{65}\text{Zn}$  absorption (Valberg et al., 1985), suggesting that this test is a valid measure of zinc absorption. However, this test does not appear to be a reliable indicator of zinc status because it can be affected by homeostatic mechanisms influencing zinc absorption (Fickel et al., 1986). The results of this test can also be influenced by factors other than absorption, including variations in gastric emptying, peripheral uptake and renal excretion (Valberg et al., 1985).

Erythrocyte zinc concentration. Studies in which erythrocyte zinc concentration has been measured as an index of zinc status have produced equivocal results. For example, a significant decrease in the mean erythrocyte zinc concentration of volunteers consuming 0.6 to 1.0 mg zinc/d for 78 days was reported by Buerk et al. (1973). Prasad et al. (1978a) also noted significant decreases in erythrocyte zinc in three of four subjects who consumed diets containing 2.7 or 3.5 mg zinc/d for two months. Conversely, Baer and King (1984), in a zinc depletion study (0.28 mg zinc/d) lasting four to nine weeks, and Rabbani et al. (1987), in a zinc stabilization-depletion-repletion study lasting 56 weeks, failed to show a change in mean erythrocyte zinc



concentrations. These apparently conflicting results might be explained once more is learned about the metabolism of zinc in erythrocytes, the dependence of erythrocyte zinc on circulating levels of zinc and the influence of factors such as the duration, severity and rapidity of onset of zinc deficiency. The lack of standardization in terms of the form of the erythrocyte sample used (i.e. washed cells, cell lysates, cell membranes, density gradient-separated cells, etc.) and the units employed to express erythrocyte zinc concentrations (i.e.  $\mu\text{g/g}$  protein,  $\mu\text{g/g}$  hemoglobin,  $\mu\text{g/ml}$  erythrocyte lysate, etc.) may also account for divergent results.

#### Leukocyte zinc and neutrophil zinc concentrations.

Several researchers have suggested that the zinc content of leukocytes (Meadows et al., 1981; 1983; Prasad et al., 1978a) and specific cellular types of leukocytes (i.e. neutrophils) (Prasad and Cossack, 1982) may be more reliable indices of zinc status than plasma/serum or erythrocyte zinc concentrations. Despite these reports, there are several methodological problems associated with the use of these techniques, and the validity of using these measures as indices of zinc status remains uncertain (Gibson, 1990).

Urinary zinc concentration. Low urinary zinc excretion was first reported in dwarfs from the Middle East suffering from severe zinc deficiency (Prasad et al., 1963a). Reduction in urinary zinc excretion has also been reported in

experimentally induced zinc deficiency (Baer and King, 1984; Hess et al., 1977). In fact, Baer and King (1984) found that urinary zinc responded more rapidly to changes in zinc intake than plasma zinc and suggested that the former might be useful for evaluating zinc nutriture.

Urinary zinc excretion also appears to be sensitive to marginal zinc intakes, although the length of time the subjects are exposed to a reduced intake, the level to which the intake is reduced and the calcium and phytate contents of the diet may affect the overall response. For example, urinary zinc losses were not reduced after nine days in subjects fed a diet containing 5.5 mg zinc/d (Wada et al., 1985), nor were they reduced after six weeks of consuming a diet containing 7.2 mg zinc/d (Thomas et al., 1992). There were, however, significant reductions in urinary zinc excretion after consuming a diet containing 3.2 mg zinc/d for six weeks (Thomas et al., 1992) and after adhering to a feeding protocol that consisted of one week of 0.6 mg zinc/d and two weeks of a diet containing 4 mg zinc/d plus additional phytate and calcium (Ruz et al., 1991).

Despite the apparent sensitivity of urinary zinc excretion to dietary zinc intake, the large variability in zinc excretion in normal healthy subjects fed a zinc adequate diet suggests that urinary zinc excretion may be affected by differences in tissue zinc status (King, 1986). This may hamper the usefulness of urinary zinc excretion as an index of

zinc status. Furthermore, urinary zinc excretion can be affected by other factors, with hyperzincuria and zinc deficiency occurring concurrently in individuals with sickle cell disease and cirrhosis of the liver. Hyperzincuria also occurs in certain renal diseases and infections and after injury, burns, acute starvation and treatment with chlorothiazide (Prasad, 1983). Consequently, urinary zinc excretion may only be useful as a measure of zinc status in apparently healthy individuals (Gibson, 1990).

Activity of zinc-dependent enzymes. Measurement of the activities of various zinc-dependent enzymes has been proposed as a way to assess zinc status. Of these enzymes, measurement of serum alkaline phosphatase activity is most common. Low serum alkaline phosphatase activity has been reported in human zinc deficiency due to acrodermatitis enteropathica (Weismann and Høyer, 1985) and in patients receiving unsupplemented total parenteral nutrition (Kay et al., 1976; Weismann and Høyer, 1985). The activity of this enzyme has been shown to increase when zinc supplements are administered to patients with these conditions (Weismann and Høyer, 1985). Similar responses have been shown to occur in experimental zinc deficiency followed by repletion (Baer et al., 1985; Prasad et al., 1978a). Conversely, Hess et al. (1977) found no consistent change in serum alkaline phosphatase activity during an experimental zinc depletion study lasting five weeks. No significant changes in serum alkaline phosphatase

activity were noted by Ruz et al. (1991) either, during experimentally-induced mild zinc deficiency and repletion. The controversial nature of these results, and the fact that the activity of this enzyme can be affected by concurrent liver/bone disease, suggest that use of serum alkaline phosphatase activity as a determinant of zinc status should be reserved for use in normal healthy subjects and should not be employed as the sole criteria for diagnosing zinc deficiency.

Angiotensin-converting enzyme is a zinc metalloenzyme that converts angiotensin I to angiotensin II. Activity of this enzyme has been reported to be lower in zinc-deficient rats than in zinc-supplemented rats and can be increased by in vitro addition of zinc, with a greater percent increase in activity occurring in zinc-deficient rats compared to controls (Reeves and O'Dell, 1985). Low angiotensin-converting enzyme activity has also been associated with low serum zinc concentrations in human subjects who have lung cancer (Bakan et al., 1988). Based on these findings, it has been suggested that determination of angiotensin-converting enzyme activity might be useful for assessing zinc status in humans; however, studies in which experimental zinc deficiency has been induced in human subjects (Milne et al., 1987; Ruz et al., 1991) do not support this idea.

Zinc content of hair. As noted by Gibson (1990), the zinc content of hair has been proposed as an index of chronic suboptimal zinc status in children, providing the confounding

effect of severe protein-energy malnutrition is absent. Support for this idea has come from studies of children with marginal zinc deficiency, characterized by low growth percentiles, who also had low hair zinc concentrations (Buzina et al., 1980; Gibson et al., 1989; Hambidge et al., 1972; Smit-Vanderkooy and Gibson, 1987; Xue-Cun et al., 1985). However, zinc supplementation was not always effective in improving hair zinc concentrations in these children, causing concern about the appropriateness of using hair zinc concentration as a measure of zinc status.

Studies comparing hair zinc concentrations to circulating levels of zinc have also produced conflicting results. For example, Klevay (1970) found a significant correlation between hair zinc and serum zinc concentrations in a group of Panamanian children. Conversely, a cross-sectional study (McBean et al., 1971) of Iranian children, and a short-term longitudinal zinc depletion/repletion study (Lane et al., 1982), did not find a positive correlation between plasma zinc and hair zinc concentrations. These discrepancies may be explained in part by the fact that the zinc content of the hair shaft reflects the quantity of zinc available to the hair follicles over an earlier time interval. Consequently, positive correlations between hair zinc and plasma zinc concentrations in children may only be evident in chronic zinc deficiency (Gibson, 1990).

The usefulness of hair zinc concentration as an indicator of suboptimal zinc status in adults is questionable. Mean hair zinc concentration was not affected in response to experimentally-induced acute zinc depletion (Baer and King, 1984; Ruz et al., 1991). Conversely, low hair zinc concentrations have been reported in individuals who have chronic diseases that are associated with impaired zinc status such as sickle cell disease (Prasad et al., 1975; 1976) and acrodermatitis enteropathica (Amador et al., 1975), but this finding was not consistent among patients with Crohn's disease (Solomons et al., 1977). Physiological state may also affect the hair zinc concentration since some (Hambidge and Droegemueller, 1974; Vir et al., 1981), but not all (Campbell-Brown et al., 1985; Hambidge et al., 1983), researchers have reported a decrease in hair zinc concentration during gestation. Other confounding factors include environmental contaminants, gender, age, season, hair color, hair treatments, rate of hair growth and rate of zinc delivery to the hair root (Hambidge, 1982).

### Metallothionein

As suggested by the foregoing discussion, a single reliable method for diagnosing zinc deficiency and assessing zinc reserves has not been identified. Two new methods that may be useful indicators of zinc status in humans are the measurement of plasma and erythrocyte metallothionein

concentrations (Golden, 1989). A brief review of the structure, function and regulation of metallothionein, followed by a discussion of the potential usefulness of erythrocyte and plasma metallothionein concentrations for assessment of zinc status, are presented in the succeeding paragraphs.

Metallothionein is the term used to refer to a family of low molecular weight, cytosolic proteins that are capable of binding heavy metals such as cadmium, copper and zinc. These proteins are single-chain polypeptides containing 60 to 61 amino acids, with cysteine as the predominate amino acid residue. Other features of the primary structure include the fact that the native protein contains no disulfide bonds, histidine residues or aromatic amino acids. The molecular weight of mammalian metallothionein, as determined from sequence data, is about 6000 Daltons for the native protein, although the actual molecular weight can range from 6500 to 7000 Daltons depending on the metal composition (Dunn et al., 1987).

The physiological significance of metallothionein has not been clearly identified; however, proposed functions of this protein include homeostatic and cytoprotective roles. Potential cytoprotective roles include detoxification of heavy metals, free radical scavenging and protection against ultraviolet light and X-ray damage (Dunn et al., 1987). One example of a homeostatic function is the ability of intestinal

metallothionein to block zinc absorption when zinc intake is high (Cousins, 1985). Metallothionein may also regulate the movement of zinc within cells and may participate in ligand-exchange reactions by donating ions needed for activation of metalloenzymes and/or zinc-requiring domains of DNA-binding factors (i.e. zinc fingers) (Cousins and Hempe, 1990; Dunn et al., 1987).

Although metallothionein isoforms have been isolated from most vertebrate tissues, they are particularly abundant in the liver, kidney and intestine. Very low concentrations of metallothionein isoforms have been found in plasma, urine and bile. The major isoforms of metallothionein, metallothionein-1 and metallothionein-2, are found in most vertebrate tissues. Expression of these isoforms is influenced by species, tissue type, physiological state and exposure to metals (Dunn et al., 1987). Although the predominant form of metallothionein is metallothionein-2 (Dunn et al., 1987), a recent study by Huber and Cousins (1993) showed that the primary gene expressed in the bone marrow of rats is metallothionein-1. The predominate form in human bone marrow/erythrocytes has not been determined.

Metallothionein biosynthesis in the liver and intestine is influenced by the zinc status of the animal, with low concentrations in zinc-deficient animals and increased quantities following zinc repletion (Richards and Cousins, 1976). Zinc has been shown to induce metallothionein



synthesis in rats by increasing the rate of metallothionein gene transcription (Blalock et al., 1988). Evidence for metallothionein induction by zinc in humans has also been obtained (Grider et al., 1990). The proposed mechanism involves binding of the metal to a nuclear regulatory factor which subsequently binds to the DNA sequence of the metal regulatory element (Cousins et al., 1988; Hamer, 1986). Evidence supporting this hypothesis was recently reported by Cousins and Lee-Ambrose (1992). These researchers demonstrated that nuclear zinc uptake and metallothionein gene expression are influenced proportionately by the level of dietary zinc intake in rats, and that this newly acquired zinc binds to nuclear zinc-binding factors, one of which appears to bind to one of the known metal regulatory element sequences.

In addition to metallothionein induction by metal ions, hepatic metallothionein synthesis is also influenced by hormones (i.e. glucocorticoids, glucagon and epinephrine), as well as other factors, such as cyclic adenylyl monophosphate, interferon, interleukin-1, food restriction and tissue injury (Dunn et al., 1987). The exact mechanisms whereby each of these factors affect metallothionein synthesis have not been fully delineated, but there is significant tissue specificity.

The concentration of metallothionein in the plasma has been shown (Mehra and Bremner, 1984) to correlate with dietary zinc intake in neonatal rats and is a reflection of changes in the concentration of hepatic metallothionein.

Sato et al. (1984) showed that plasma and hepatic metallothionein concentrations were reduced to nondetectable levels in zinc-deficient rats, but increased in adequately nourished rats exposed to stress or infection. This is in contrast to plasma zinc concentrations which are reduced in response to both a decrease in the size of the exchangeable zinc pool and in response to certain metabolic conditions (i.e. stress, infection and/or hormones). Based on these findings, it was proposed (Golden, 1989; King, 1990) that plasma metallothionein concentrations could be used to differentiate between low plasma zinc concentrations occurring in response to a reduction in the size of the exchangeable zinc pool, versus low concentrations occurring in response to metabolic conditions. Accordingly, low plasma zinc and low plasma metallothionein concentrations would suggest a reduction in the size of the exchangeable zinc pool due to low zinc intakes; whereas, elevated plasma metallothionein and low plasma zinc concentrations would suggest that tissue zinc is being redistributed in response to factors other than zinc deficiency (Golden, 1989; King, 1990). A potential problem associated with the use of this approach to diagnose zinc deficiency is that plasma metallothionein concentrations in zinc-deficient animals subjected to stress may be close to those of normal animals (Sato et al., 1984), making it difficult to differentiate between these conditions. A second problem is that plasma metallothionein concentrations are

difficult to measure, especially in adult animals, because concentrations are close to the detection limit of most immunoassays (Bremner et al., 1987).

Recognition of the potential problems associated with the use of plasma metallothionein concentrations to diagnose zinc deficiency stimulated interest in determining if other body fluids might be appropriate indicators of zinc status. Animal studies (Bremner et al., 1987) showed that erythrocyte metallothionein concentrations were very sensitive to changes in dietary zinc but not copper or selenium supply, were not affected by stress or infection and were at least ten-fold higher than plasma metallothionein concentrations. These findings resulted in the idea that the erythrocyte metallothionein concentration might be a sensitive, reliable and more convenient method for assessing zinc status.

A human metallothionein enzyme-linked immunosorbent assay (ELISA) has been developed (Grider et al., 1989) and tested (Grider et al., 1990; Thomas et al., 1992). Grider et al. (1990) showed that erythrocyte metallothionein concentrations were very responsive to changes in zinc intake in human subjects. Erythrocyte metallothionein concentrations fell to 68% of the initial value after 6 days of consuming a zinc-deficient diet (~0.5 mg/d), whereas fasting plasma zinc concentrations were only reduced by 7%. Conversely, supplementation with 50 mg zinc/d resulted in a seven-fold increase in erythrocyte metallothionein concentrations in just

seven days. Thomas et al. (1992) confirmed the responsiveness of erythrocyte metallothionein to zinc-deficient intakes in human subjects and also found that comparisons of the change in erythrocyte metallothionein concentrations in subjects fed graded levels of zinc for six weeks could be used to distinguish between low and adequate levels of dietary zinc intake. Thus, it appears that the concentration of metallothionein in erythrocytes can be used as a sensitive and reliable indicator of zinc status in humans under controlled dietary conditions.

### Zinc Deficiency

Zinc deficiency in humans was first documented in the early 1960s (Prasad et al., 1961; 1963b) in Egyptian and Iranian male adolescents who were consuming vegetable protein-based diets. The symptoms observed in these young men included retarded growth, delayed sexual development, hypogonadism, rough skin, severe anemia and lethargy. Other symptoms frequently associated with zinc deficiency include: anorexia, alopecia, reduced taste acuity, emotional disorders (e.g. depression; irritability), diarrhea, delayed wound healing and impaired immune function (Aggett, 1989; Hambidge et al., 1986; Solomons, 1988).

The development of zinc deficiency is thought to occur in progressive stages arbitrarily referred to as mild and severe (Hambidge, 1989). The occurrence of severe zinc deficiency,

which is relatively uncommon, is usually associated with diseases such as acrodermatitis enteropathica or malabsorption syndromes (Aggett, 1989). Cases of severe zinc deficiency have also been documented in patients receiving unsupplemented total parenteral nutrition (Arakawa et al., 1976; Kay et al., 1976). Although the clinical features of zinc deficiency are relatively nonspecific, severe zinc deficiency is usually associated with a complex of symptoms that signal the possibility of a zinc-deficient state (Aggett, 1989; Hambidge et al., 1986). These symptoms include dermatitis, neuropsychiatric changes, diarrhea, alopecia, anorexia and weight loss. Plasma zinc concentrations are usually depressed as well. The response to increased zinc intake and/or supplementation is usually rapid and is taken as confirmation of the diagnosis (Hambidge et al., 1986). Untreated zinc deficiency results in death (Prasad, 1991).

The lack of a sensitive and specific laboratory index of zinc deficiency, as well as the lack of specific clinical features, have hampered the ability to detect mild human zinc deficiency (Hambidge, 1989). Despite these problems, there is consensus that mild zinc deficiency of dietary origin or resulting from altered zinc metabolism/requirements occurs in humans. Population groups considered to be most at risk for mild zinc deficiency include infants, children and pregnant women, as well as individuals with diseases such as Crohn's disease, cystic fibrosis, sickle cell disease and insulin-

dependent diabetes mellitus. Symptoms suggestive of mild zinc deficiency include impaired immune function, neurosensory changes and, in infants and children, a reduction in growth rate and/or the quality of growth (Hambidge, 1989).

### Folate-Zinc Interactions

There are two main concerns with regard to folic acid-zinc interactions. The first concern is related to the role of zinc in folate absorption and metabolism; the second is related to the potential adverse effect of supplemental folic acid on zinc absorption and zinc status. A review of the research designed to investigate these relationships is presented in the following sections of this dissertation.

#### Effect of Impaired Zinc Status on Folate Absorption and Metabolism

One of the first reports suggesting a relationship between impaired zinc status and folate absorption and/or metabolism was prepared by Williams and Mills (1973). These researchers observed a reduction in hepatic folate concentration in rats fed zinc-deficient diets, although a significant change in serum folate concentration was not detected. This report, as well as a study showing that bovine hepatic folate conjugase (Silink et al., 1975) was a zinc-dependent enzyme, provided the basis for subsequent experiments designed to investigate the effect of impaired zinc status on folate absorption and metabolism.

Several researchers have investigated the effect of dietary zinc deficiency on intestinal mucosal and/or pancreatic folate conjugase activity and absorption of pteroylpolyglutamates in rats. Canton et al. (1989) reported significantly reduced folate conjugase activities in pancreatic tissue and intestinal luminal wash in zinc-deficient versus zinc-adequate rats. Zinc-deficient rats also had significantly reduced plasma folate concentrations. Significantly reduced pancreatic folate conjugase activities and plasma folate concentrations were noted in subsequent studies of similar design (Canton and Cremin, 1990; Hewedy et al., 1991).

Recently, Tamura and Kaiser (1991) measured intestinal mucosal folate conjugase activity, using intestinal mucosal homogenates, and absorption of [ $^{14}\text{C}$ ]PteGlu<sub>7</sub> and [ $^3\text{H}$ ]PteGlu in rats fed a zinc-deficient diet. No significant differences were found in intestinal mucosal folate conjugase activity or absorption of [ $^{14}\text{C}$ ]PteGlu<sub>7</sub> or [ $^3\text{H}$ ]PteGlu in rats fed the zinc-deficient diet compared with zinc-supplemented control rats. These researchers concluded that intestinal mucosal folate conjugase is not zinc-dependent in rats and that zinc deficiency does not impair intestinal absorption of polyglutamates.

As noted by Tamura and Kaiser (1991), the discrepancy between the results of their study and the former studies may be attributed to the following: differences in the strain of

rats used (i.e. Wistar versus Sprague-Dawley); differences in the methods used to assess folate absorption (i.e. measurement of plasma folate concentrations for 3 h after the folate dose versus measurement of 24 h urinary excretion and fecal loss of radioactivity); the source of pteroylpolyglutamate (i.e. polyglutamates derived from yeast, which may have contained folate hydrolase inhibitors, versus synthetic [ $^{14}\text{C}$ ]PteGlu) and; inclusion versus exclusion of saturating doses of folic acid prior to the oral tests. Regardless of the underlying explanation(s) for the discrepancies between these studies, the significance of these results to humans is questionable since rats exhibit little or no brush border folate conjugase activity (Wang et al., 1985), and the contribution of human pancreatic folate conjugase to the hydrolysis and absorption of pteroylpolyglutamates is uncertain (Bhandari et al., 1990; Jagerstad et al., 1976).

Tamura et al. (1978) examined the effect of severe zinc depletion on the absorption of folic acid in human subjects by measuring serum folate concentrations after oral administration of pteroylmonoglutamate and pteroylpolyglutamate. They found that zinc deficiency adversely affected absorption of pteroylpolyglutamate, but not pteroylmonoglutamate. Enzyme activity was not directly measured, and no attempt was made to determine whether there was a general or intestinal decrease in protein and DNA synthesis in zinc deficiency; however, based on the findings



of Silink et al. (1975), they hypothesized that zinc was essential for maintaining normal activity of intestinal conjugase in humans. The results of subsequent studies (Day and Gregory, 1984; Gregory et al., 1987; Wang et al., 1985) showing that human intestinal brush border folate conjugase is indeed zinc-dependent support this hypothesis.

No other human studies designed to confirm the findings of Tamura et al. (1978) or to study the effect of marginal zinc intakes on folate absorption and utilization over time have been conducted. Since marginal zinc intake/deficiency is more prevalent than severe zinc deficiency (Hambidge, 1989), research directed at studying the effect of marginal zinc intakes on folate utilization would appear to be more relevant than studies of individuals with severe zinc deficiency.

In addition to the potential adverse effect of zinc deficiency on the hydrolysis and absorption of folate, it is possible that a deficiency of this nutrient may indirectly alter folate metabolism. Tamura et al. (1987) found that hepatic methionine synthetase activity was significantly higher in zinc-deficient rats compared with zinc-adequate control rats (i.e. pair-fed and ad libitum), while hepatic  $N^{5,10}$ -methylene-tetrahydrofolate reductase activity was similar in all groups. Other changes noted included decreased total liver and plasma folate concentrations and significantly greater oxidation of formate and histidine in zinc-deficient rats, suggesting that the amount of available non- $N^5$ -methyl-

tetrahydrofolate increases in zinc deficiency. Based on these findings, Tamura et al. (1987) hypothesized that increased methionine synthetase activity associated with zinc deficiency in rats may regulate the tissue distribution of folate coenzymes. Whether or not these changes occur in humans has not been determined, but the concept that impaired zinc status may indirectly alter folate metabolism is intriguing.

#### Effect of Supplemental Folic Acid on Zinc Status

A review of the research related to the question of the effect of supplemental folic acid on zinc status is presented below. Studies suggesting a negative effect are reviewed first, followed by those suggesting no effect. The results of a new study examining this issue are presented in Chapter 5.

The suggestion that consumption of modest amounts of supplemental folic acid might have an antagonistic effect on zinc status was first presented by Milne et al. (1984). These researchers examined the effect of supplemental folic acid in eight men fed 7.5 mg zinc/d for 4 weeks, 3.5 mg zinc/d for 16 week and 33.5 mg zinc/d for 4 weeks. The folate content of the diet was 150-180  $\mu\text{g}/\text{d}$ . Supplemental folic acid (400  $\mu\text{g}$  every other day) was provided to the same four subjects during each diet period. Plasma zinc concentrations were not significantly different between either of the folate treatment groups at any level of zinc intake; however, the folic acid supplemented group had significantly higher fecal zinc and

significantly lower urinary zinc losses when their zinc intake was either 7.5 or 3.5 mg/d. No significant difference in fecal zinc losses was observed when zinc intake was 33.5 mg/d. Net zinc balance was not significantly different between the treatment groups at any level of zinc intake. Despite the lack of a significant effect on zinc balance, the authors concluded that supplemental folic acid influences zinc homeostasis and that the mechanism may involve the formation of an insoluble chelate and impairment of absorption. Interestingly, there were no changes between the treatment groups in their excretion patterns of iron and copper; minerals with which folic acid is known to form stable complexes.

In an observational study of 450 pregnant women, Mukherjee et al. (1984) noted a significant association between the occurrence of pregnancy complications and the combination of low maternal plasma zinc and high maternal plasma folate concentrations. This combination of maternal blood values was also associated with the occurrence of fetal distress. These investigators speculated that, in addition to iron, folic acid present in prenatal vitamin/mineral supplements might inhibit the intestinal absorption of zinc causing impaired zinc status and the development of fetomaternal complications.

Using a series of in vivo and in vitro rat experiments, Ghishan et al. (1986) examined the relationship between zinc

and folic acid. In one of these experiments, 30 cm segments of rat small bowel were perfused in situ with a solution containing folic acid and  $^{65}\text{Zn}$  with a zinc to folic acid molar ratio of 1:4.5. Mucosal uptake of the labeled zinc was significantly diminished when folic acid was present in the lumen. The effect of folic acid on zinc appeared to be related to the presence of zinc and folic acid together in the intestinal lumen because parenteral administration of folic acid had no effect on zinc transport. The significance of these findings to humans is questionable because the amount of folic acid administered to the animals resulted in a zinc to folic acid ratio that greatly exceeded that which would be obtained with commonly prescribed levels of folic acid supplementation.

Ghishan et al. (1986) also studied the effect of zinc on folic acid absorption using an in vitro preparation of everted rat jejunal segments. Mucosal-to-serosal transport of  $0.1\ \mu\text{M}$  and  $0.5\ \mu\text{M}$  radio-labeled folic acid was significantly decreased in the presence of zinc chloride at concentrations of 250 and  $500\ \mu\text{M}$ . In an attempt to understand the nature of this mutual inhibitory effect, these investigators conducted in vitro charcoal binding studies. They found that zinc and folic acid formed insoluble complexes at pH 2.0, but at pH 6.0, these complexes dissolved. Since binding did not occur at the normal pH of the intestine (i.e.  $\sim$  pH 6.0), it was concluded that under normal physiological conditions the site

of the mutual inhibitory effect between zinc and folic acid must occur at the intestinal membrane level.

Plasma zinc response to folic acid supplementation was determined by Simmer et al. (1987), using the oral zinc tolerance test, in ten pregnant women before and after two weeks of daily supplementation with 100 mg iron and 350  $\mu$ g folic acid. Ten nonpregnant subjects supplemented with 350  $\mu$ g folic acid/d for two weeks were also studied. The oral zinc tolerance test was conducted using 25 and 50 mg zinc loads for the pregnant and nonpregnant subjects, respectively. The areas under the plasma zinc concentration-time curves and the peak heights of the curves were significantly decreased after supplementation in both groups of subjects. Whether or not a similar response would have been observed with a lower dose of zinc sulfate or with dietary zinc is unknown. The fact that there was a 24 h interval between the period of supplementation and reassessment of zinc absorption makes it unlikely that zinc absorption was impaired by the formation of an insoluble folic acid/iron-zinc chelate in the lumen, as suggested by Milne et al. (1984), and more likely that the reported effect occurred at the level of the intestinal membrane as proposed by Ghishan et al. (1986). As discussed in an earlier section, use of the oral zinc tolerance test as a tool for assessing the effect of other nutrients on zinc absorption has been criticized because high levels of oral zinc are used to produce the response and the test can be

influenced by factors other than absorption (Valberg et al., 1985).

To circumvent some of the problems associated with the oral zinc tolerance test, Milne (1989) examined the effect of folic acid on zinc absorption by determining absorption of a tracer dose of  $^{65}\text{Zn}$  from a breakfast meal. The percent of labeled zinc absorbed from the meal with and without 800  $\mu\text{g}$  folic acid was determined in 13 subjects (seven men and six women) at weekly intervals using a whole-body counter. The mean percent absorption of  $^{65}\text{Zn}$  was not significantly different during the control and folic acid supplemented periods. However, when subjects were divided into two groups (i.e. those with control zinc absorption above and below 30%), only the subjects with control zinc absorption above 30% experienced a significant reduction in zinc absorption when folic acid was fed with the meal.

In a separate study, the same subjects ingested 800  $\mu\text{g}$  folic acid daily for two weeks. At the end of the supplementation period they were fed the same  $\text{Zn}^{65}$ -labeled breakfast meal used for the previous study. Plasma zinc and serum and erythrocyte folate concentrations were determined before and after two weeks of folic acid supplementation, and whole-body counting was performed at weekly intervals. Similar to the results of the first study, the mean percent zinc absorption after daily folic acid supplementation was not significantly different from the control values unless the

subjects were divided into groups with control absorption values above or below 30%. The subjects with control absorption values greater than 30% had significantly lower absorption values when they were receiving the supplement; no effect was seen in the subjects with low control absorption values. Plasma zinc and serum and erythrocyte folate values were not significantly different between subjects with high versus low control absorption values, nor was there a significant difference in zinc turnover between the control and supplemented periods. Although these studies suggested that supplementation with folic acid did not impair zinc absorption, Milne (1989) proposed that an interaction between zinc and folic acid may only be manifested in conditions of increased zinc need or low zinc intake.

Milne et al. (1990) confirmed the lack of an effect of supplemental folic acid (i.e. 400 and 800  $\mu\text{g}/\text{d}$ ) on zinc balance, zinc absorption and static indices of zinc status in men consuming a zinc-adequate diet (i.e. 12.5 mg zinc/d). However, folic acid supplementation impaired the mobilization of zinc into the plasma following participation in continuous, graded maximal exercise. It was suggested that in contrast to the effects of supplemental folic acid in subjects consuming a zinc-restricted diet (Milne et al., 1984), subjects consuming a zinc-adequate diet could maintain zinc homeostasis when given supplemental folic acid, but this homeostatic mechanism was insufficient to prevent changes in zinc

mobilization during graded maximal exercise (Milne et al., 1990).

The most recent report (Fuller et al., 1992) suggesting that supplemental folic acid may adversely affect zinc status was based on retrospective data collected from preterm infants housed in a special care unit for up to the first 16 weeks of life. The sample included 60 infants, 48 of whom received oral supplementation with 1 mg folic acid/d. Statistical analysis revealed a significant inverse relationship between the maximum serum folate level attained and the minimum serum zinc level attained for each infant. This relationship remained significant after corrections for extraneous factors such as gestational age at birth, diet, birth weight, gender, fetal growth retardation, assisted ventilation and length of time to full enteral feeding. The meaningfulness of these results is questionable, however, because the minimal plasma zinc and maximal plasma folate values used for statistical analysis did not necessarily occur on the same day. In some cases, the lowest plasma zinc values occurred before the highest plasma folate values. Additionally, there was no control group; the data for the 12 infants who did not receive folic acid supplements were analyzed together with the data for those infants who received supplementation. Other factors that could have influenced the results include the time at which oral folic acid supplementation was started, the length of time each subject received the supplement before being



discharged from the unit, and the fact that some infants received supplemental zinc and/or folic acid parenterally for different lengths of time prior to the initiation of oral folic acid supplementation.

The first rat and human experiments suggesting that supplementation with folic acid did not impair zinc absorption or utilization were conducted by Keating et al. (1987). Similar to the study by Simmer et al. (1987), Keating and his colleagues used the oral zinc tolerance test to assess zinc absorption in human subjects. In this study, the serum zinc concentration-time curve was determined for six healthy men given a 25 mg oral dose of zinc as zinc sulfate. Eight days later, the test was repeated with the addition of a 10 mg oral dose of folic acid. In contrast to the Simmer study, there were no significant differences in the areas under the serum zinc concentration-time curves or peak heights with or without 10 mg folic acid. The discrepancy between these results and the findings of Simmer et al. (1987) may be due to differences in the experimental design. It is possible that a single large dose of folic acid does not have the same effect as smaller doses taken over a period of time.

Rat experiments performed by Keating et al. (1987) examined zinc bioavailability using two different methods. In the first study, an aqueous solution containing 13.0  $\mu\text{g}$  zinc as zinc chloride plus 2  $\mu\text{Ci}$   $^{65}\text{Zn}$  or an infant formula containing the same amount of unlabeled and radio-labeled

zinc, was administered to the rats via intragastric intubation. These solutions were further supplemented with 0, 4.4 or 176  $\mu\text{g}$  folic acid. The animals were sacrificed 5 h after intubation, and the accumulation of  $^{65}\text{Zn}$  in the livers and kidneys was measured. Zinc retention by the livers or kidneys was not affected by either level of folic acid supplementation. These results are surprising if one considers that Ghishan et al. (1986) showed that zinc transport was inhibited in rats when folic acid was present in the intestinal lumen. This apparent inconsistency may be explained in part by the fact that Ghishan et al. (1986) introduced the folic acid into the intestinal lumen, whereas Keating et al. (1987) introduced the supplement intragastrically. Additionally, the molar ratios of zinc:folic acid used were 1:4.5 in the former study and 20:1 and 1:2 in the latter experiment.

In the second part of the rat study conducted by Keating et al. (1987), comparisons of total femur zinc and weight gain were made in growing male rats fed a basal diet containing no folic acid or zinc, or diets containing either 6 or 12 mg zinc/kg diet supplemented with 0, 2 or 160 mg folic acid/kg diet. The addition of 2 or 160 mg folic acid/kg diet had no significant effect on weight gain of the animals or zinc uptake by the femur at either level of zinc intake. The results of this experiment substantiate the findings of the other experiments conducted by Keating et al. (1987).

The opportunity to study the long-term effects of large oral doses of folic acid in human subjects was provided by an ongoing intervention trial designed to evaluate the possible relationships between cervical dysplasia and nutritional status in women. In this study (Butterworth et al., 1988), fifty women with cervical dysplasia were randomly assigned to receive supplementation with 10 mg/d oral folic acid or a placebo. Dietary intake was not controlled or assessed during this study. Erythrocyte folate and plasma and erythrocyte zinc were evaluated at the initial visit and after 2 months of treatment; 21 of the same subjects were evaluated after 4 months of treatment. Erythrocyte folate concentrations were significantly higher in the folic acid-supplemented subjects compared to the placebo-treated subjects after 2 and 4 months of treatment suggesting compliance with the treatment; however, there were no significant differences in plasma or erythrocyte zinc concentrations between the groups. A potential problem with the interpretation of these results is that plasma and erythrocyte zinc concentrations tend to remain fairly stable unless zinc status is severely compromised. Consequently, it is difficult to assess whether the lack of an effect of supplemental folic acid on plasma and erythrocyte zinc is due to failure of these response variables to detect the effect, or if indeed, no change occurred.

As reported in an abstract, Krebs et al. (1988) measured the absorption of  $^{70}\text{Zn}$  administered with and without 30 mg of

folic acid in three healthy subjects. Similar to the findings of Keating et al. (1987), this pharmacologic dose of folic acid did not impair zinc absorption. These researchers also reported that mean plasma, mononuclear and neutrophil zinc, as well as serum alkaline phosphatase, erythrocyte delta-amino levulinic acid dehydratase and prealbumin values of patients with fragile X syndrome treated with  $16 \pm 5$  mg folic acid/d for 1 to 4 y, were not significantly different from values for healthy control subjects. Thus, zinc status did not appear to be affected by chronic ingestion of large doses of folic acid in subjects with fragile X syndrome. Although it may be inappropriate to make inferences from these data to a normal population, this study appears to corroborate the study conducted by Butterworth et al. (1988).

Fuller et al. (1988) were the first investigators to examine the effect of supplemental folic acid in rats during pregnancy and lactation. At 110 d of age, the rats were allocated to receive a diet containing the four possible combinations of: no additional folic acid or 100  $\mu$ g folic acid/g diet and 6.6  $\mu$ g zinc/g diet or 20.2  $\mu$ g zinc/g diet. The animals were acclimated to the diet for 21 d, after which they were mated and further randomized to the pregnancy or lactation study. Pups and dams were sacrificed on day 20 of gestation or day 20 of lactation. Blood samples were analyzed for plasma zinc, and maternal and pup livers and kidneys were analyzed for zinc content. Regardless of the level of zinc in

the diet, supplemental folic acid did not compromise plasma zinc or tissue zinc concentrations of pregnant rats, their fetuses, lactating rats or suckling rats. The researchers concluded that if these results are applicable to humans, then some reassurance is provided that prenatal folic acid supplementation does not necessarily cause zinc depletion in pregnant women.

A subsequent experiment conducted by an independent group of researchers (Southon et al., 1989) examined the effect of supplementation with folic acid, calcium and iron in pregnant and nonpregnant rats receiving either a low (8  $\mu\text{g/g}$ ) or high (60  $\mu\text{g/g}$ ) zinc diet. This diet was fed for 14 d before mating. After mating, half of the rats in each of the two diet groups were supplemented with iron, calcium and folic acid. On the eighteenth day of gestation,  $^{65}\text{Zn}$  was fed along with the usual diet. The animals were sacrificed on day 20 of gestation. Although whole body radioactivity was significantly lower in all groups fed the supplemented diet compared with the unsupplemented groups, and plasma zinc concentrations in the pregnant supplemented rats were significantly lower than their unsupplemented counterparts, supplementation had no effect on total femur or liver zinc content in any of the groups. Fetal zinc concentration was not affected by supplementation either. The authors concluded that the risk of inducing either maternal or fetal zinc depletion as a consequence of increased intakes of these

nutrients is very slight, since the differences in maternal zinc status due to supplementation were small, and the fetuses were apparently protected. Even if the authors had concluded that supplementation with these nutrients had an adverse effect during pregnancy or on pregnancy outcome, it would be difficult to separate the potential contribution of folic acid from that of iron and/or calcium.

Quinn et al. (1990) also used pregnant rats to study the effects of supplemental folic acid on maternal and fetal zinc status, pregnancy outcome and the incidence of fetal malformations. In this study, pregnant rats were fed a zinc-deficient ( $< 0.5$  mg zinc/kg diet) or zinc-supplemented diet (75 mg zinc/kg diet) from mating until the eighteenth day of pregnancy. Half of the rats in each zinc group received the basal level of folic acid provided by the diet (0.56 mg/kg diet). The remaining rats were supplemented with 200 mg folic acid/kg diet. Maternal plasma zinc and tibia zinc, placental zinc, litter size, fetal weight and placental weight were not significantly different in the folic acid-supplemented group compared to the unsupplemented groups within the level of zinc intake. Within the zinc-deficient groups, placental zinc content was actually significantly higher in the folic acid-supplemented animals. Examination of the fetuses for ten different types of malformations revealed a significantly higher incidence of clubbed foot in the fetuses from animals fed the zinc-deficient folic acid-supplemented diet compared

to those from the dams fed the zinc-deficient unsupplemented diet. Folic acid supplementation did not increase the incidence of other deformities. Thus with the exception of the potential influence on the development of clubbed foot, this inordinately high level of folic acid supplementation did not appear to adversely affect zinc status or pregnancy outcome. The fact that none of the fetuses from rats fed the zinc-supplemented diets (i.e. folic acid-supplemented or unsupplemented) developed clubbed foot suggests that expression of this developmental abnormality may be sensitive to folic acid supplementation only when zinc deficiency is present. This idea is congruent with Milne's (1989) hypothesis that an interaction between supplemental folic acid and zinc may only be manifested in conditions of zinc need or low zinc intake.

Pigs have also been used as an animal model to study the effect of supplemental folic acid. This animal model may be better suited for investigating folic acid-zinc interactions in humans because of the similarities between pigs and humans in the enzymatic mechanism of absorption (Day and Gregory, 1984; Gregory et al., 1987; Wang et al., 1985). Tremblay et al. (1989) studied the effect of supplemental folic acid in sows from weaning to day 30 of gestation. The sows were fed a commercial diet with or without supplemental folic acid (0 or 5 mg/kg diet). Serum folate, zinc, copper and iron were measured at weaning, mating and day 30 of gestation. Folic

acid supplementation had no effect on serum copper and iron; however, serum folate and plasma zinc concentrations were significantly elevated between weaning and day 30 of gestation in the supplemented group. Although this study suggests that folic acid supplementation did not interfere with zinc status, one must be careful in making conclusions since serum zinc concentrations were used as the sole measure of zinc status.

To study the effect of folic acid on zinc uptake in pigs, Turnbull et al. (1990) conducted an in vitro study in which intestinal brush border membrane vesicles from porcine small bowel were exposed to an incubation medium containing various concentrations of folic acid (0.05, 0.5, 5.0 and 50.0  $\mu\text{M}$ ) and a  $^{65}\text{Zn}$  concentration of 5  $\mu\text{M}$ . Uptake of  $^{65}\text{Zn}$  was not significantly changed by the addition of folic acid at any of the concentrations tested. Although this study did not detect a luminal interaction between zinc and folic acid, the possibility that a mucosal effect occurred, as suggested by Ghishan et al. (1986) and Simmer et al. (1987), was not ruled out.

Tamura et al. (1992) have recently published a study in which they examined the relationship between pregnancy outcome and maternal serum folate and zinc concentrations. These investigators measured serum folate and zinc concentrations at 18 and 30 weeks gestation and correlated these data with birth weight and Apgar scores of newborn infants and with the incidence of maternal infections during the perinatal period.



The study population consisted of 285 women who delivered full-term infants. Women with risk factors and a growth retarded infant were matched to women with a normal-sized infant by race, sex of infant, smoking status and a number of other variables. All women had been offered daily folic acid supplements of 1 mg/d during their pregnancy. Overall compliance was estimated to be approximately 48% based on serum folate concentrations of 46 nmol/L. Dietary intake was not monitored. In contrast to the study by Mukherjee et al. (1984), Tamura and associates (1992) did not find an association between high serum folate and low serum zinc concentrations. In fact, high serum folate concentrations were associated with favorable effects on pregnancy outcome. Thus, this study did not support the idea that supplementation with folic acid has an adverse effect on maternal zinc status and pregnancy outcome.

Tamura et al. (1992) have also recently reported the results of an observational study in which they measured zinc and folate concentrations in amniotic fluid. Samples of amniotic fluid were obtained during the second trimester of pregnancy from 221 women who gave birth to apparently healthy infants and 8 women who delivered infants with neural tube defects. Folate and zinc concentrations of amniotic fluid from women who delivered infants with neural tube defects were not significantly different from those of women who delivered normal infants. These researchers also reported that there

were no significant differences in amniotic fluid nutrient levels between women who took vitamin and/or mineral supplements during pregnancy and those who did not. Although the concentration of nutrients in amniotic fluid may not necessarily reflect the concentrations found in fetomaternal tissues, the results of this study may provide further evidence for the lack of a folic acid-zinc interaction.

The fact that supplemental folic acid is frequently prescribed for individuals undergoing chronic hemodialysis prompted Reid et al. (1992) to examine zinc status in a small sample of men (12) and women (9) who had been receiving hemodialysis treatments for at least 6 months. The subjects were classified into the following four groups based on their prescribed supplementation regimen: no folic acid or zinc; no folic acid and 22.5 mg zinc/d; 5 mg folic acid/d and no zinc; or 22.5 mg zinc/d and 5 mg folic acid/d. Average daily food folate and zinc intakes were estimated using a food frequency questionnaire. Serum and erythrocyte folate and serum zinc concentrations were also measured. There were no significant differences in dietary folate or zinc intakes between the groups. Erythrocyte folate concentrations were significantly higher in the folate-supplemented groups, but serum zinc concentrations were unaffected by any combination of supplementation. Although no evidence of an adverse effect of folic acid on zinc status was noted in this study, generalization of these findings is limited because of the

descriptive nature of the study, the use of a chronically-ill population and the question of the validity of serum zinc as a valid index of zinc status in these subjects.

In summary, the results of animal and human studies investigating the effect of folic acid supplementation on zinc status are equivocal. The diversity of research designs and protocols used to investigate this question may explain, to some extent, the discrepant results. Within the human subjects studies alone, factors that may have contributed to the disparity in outcomes include: differences in the metabolic states of the subjects (i.e. healthy, chronically ill, pregnant, etc.); administration of widely varying levels of supplemental folic acid; failure to control dietary intake in most studies; and the lack of a satisfactory index to assess zinc status. Milne's (1989) hypothesis that folic acid-zinc interactions may only be manifested in conditions of increased zinc need or low zinc intake may also explain the contradictory results, since most studies did not control for differences in zinc intake or prior status.

### CHAPTER 3 RATIONALE FOR RESEARCH PROTOCOL

It is apparent from the literature review pertaining to folic acid-zinc interactions that neither the question of the effect of impaired zinc status on the utilization of folic acid, nor the question of the effect of supplemental folic acid on zinc status in human subjects, has been adequately resolved. The possibility that folic acid supplementation may interfere with zinc absorption and status is disconcerting because supplementation with this vitamin is frequently recommended for many population groups, including: patients with various gastrointestinal/malabsorption syndromes; cancer patients receiving anti-folate medications; users of oral contraceptive agents; individuals with cervical dysplasia; renal patients undergoing hemodialysis treatments; and the majority of prenatal patients. Folic acid supplements may also be recommended to individuals with documented deficiencies such as the elderly. Use of supplemental doses of this nutrient is likely to become even more widespread as a result of recent recommendations suggesting that women of childbearing age who are capable of becoming pregnant increase their intake of folic acid (Anon., *Morbidity and Mortality Weekly*, 1992). These recommendations were prompted by the

results of studies demonstrating the positive correlation between the use of folic acid supplements during the periconceptional period and a reduction in the risk of occurrence/recurrence of neural tube defects. Beyond supplementation, if the Food and Drug Administration's, Food Advisory Council's recommendation to enrich flour with folic acid is accepted (Anon., *Food Chemical News*, 1993), the majority of the population will be exposed to higher intakes of this nutrient. Consequently, research directed at resolving the issue of whether folic acid supplementation exerts a deleterious effect on zinc status, and if so, under what conditions, is very timely and warranted. Impaired zinc status due to injudicious recommendation/use of folic acid supplements may have untoward consequences given the multiplicity of physiologic functions associated with zinc. Likewise, considering the central role of folate in DNA synthesis and one-carbon metabolism, impaired utilization of folate due to zinc deficiency or marginal zinc intake may also have damaging consequences.

In an attempt to provide more definitive information about the interrelationship between folic acid and zinc, the present study was designed to determine if supplemental folic acid affected zinc status in human subjects consuming zinc-adequate or zinc-restricted diets; and to determine if utilization of supplemental folic acid was affected in human subjects consuming either a zinc-restricted or zinc-adequate

diet. To overcome some of the limitations of previous studies, the subjects were fed a constant diet containing 14.5 or 3.5 mg zinc/d for two, 28 day study periods. The study periods were separated by an 80-day washout period. During the study periods, subjects were assigned to receive supplemental folic acid and placebo treatments in a crossover fashion, so that subjects served as their own control on and off the supplement. Supplementation was provided at a level comparable to that used in many prenatal vitamin supplements. In addition to traditional measures used to assess zinc status, erythrocyte metallothionein concentrations were evaluated as a new and potentially more responsive index of zinc status (Grider et al., 1990). The responsiveness of erythrocyte metallothionein concentrations to dietary zinc intake has been confirmed more recently by Thomas et al. (1992).

The practical constraints of conducting a feeding study (i.e. space, facilities, meal preparation, cost, etc.) made it necessary to limit the number of subjects who could be included in each treatment group. To minimize heterogeneity within this small group of subjects, the study was restricted to Caucasian males. The racial restriction was imposed because differences in folate status and hematological parameters have been observed in population studies (Bailey 1982a; 1982b). Males were selected over females because erythrocyte metallothionein concentrations have not been

measured or standardized for this group. Also, previous feeding studies examining the effect of folic acid supplementation on zinc status in subjects fed a zinc-restricted (Milne et al., 1984) or zinc-adequate (Milne, 1989) diet included only male subjects. Thus, by limiting the present study to males, the potential question of whether differences in outcome measures were due to gender differences was avoided.

Data from the study conducted by Grider et al. (1990) were used to determine the length of the study periods. These researchers found a significant decrease in erythrocyte metallothionein concentrations within seven days after consuming a diet containing less than 1 mg zinc/d. Since the response to a diet containing 3.5 mg zinc/d had not yet been determined, it was assumed that a longer period of time might be needed to observe any changes due to the effect of supplemental folic acid. Consequently, the study periods were increased by a factor of 3.5. This also provided the opportunity to examine erythrocyte metallothionein response over a longer time interval.

The length of the washout phase of the study was planned using data from Heseker and Schmitt (1987). These researchers determined the average daily increase in erythrocyte folate concentrations after 17 weeks of supplementation with 1 mg folic acid/d, as well as the average daily rate of decline after cessation of supplementation. Based on these

calculations, it was estimated that an 80-day washout period would be more than sufficient to eliminate any residual effects due to folic acid supplementation.

As intimated by the objectives outlined above, this study was also designed to take into account the possibility that folic acid-zinc interactions may only be manifested in conditions of low zinc intake. This was achieved by randomly assigning the subjects to either a zinc-restricted diet (3.5 mg zinc/d) or an identical diet supplemented with 11 mg of zinc as zinc sulfate (14.5 mg zinc/d total intake). A zinc-restricted diet, rather than a zinc-deficient diet, was selected because this level of intake is more representative of the zinc content of diets consumed by individuals with marginal zinc intakes and/or status.

Another unique aspect of this study was the use of deuterium-labeled folic acid monoglutamate to examine the effect of zinc-adequate and zinc-restricted diets on folate utilization over time. The availability of stable isotopically-labeled folic acid provided the opportunity to quantify the excretion of supplemental folic acid without the risks associated with the use of radio-isotopes in human subjects.



## CHAPTER 4 SUBJECTS, EXPERIMENTAL DESIGN, MATERIALS AND METHODS

### Subjects

#### Recruitment and Selection

The University of Florida Institutional Review Board approved the screening and experimental protocols for this study, and informed consent was obtained from all prospective subjects. Subjects were recruited from the University of Florida campus and the city of Gainesville through advertisements. Interested individuals completed a questionnaire (Appendix A) and food records (Kauwell Methodology, 1993, Food Science and Human Nutrition Archives) which were evaluated by the investigator to determine eligibility. Fifty of the original prospects were selected for further screening, which included an interview with the investigator and donation of a blood sample for routine laboratory analysis (i.e. 25-item blood chemistry profile and a complete blood count with differential).

Subjects were selected after reviewing the information obtained from the questionnaires, food records, interviews and blood chemistry profiles. This information was used to select Caucasian males between the ages of 20 to 35 with no history

or current diagnosis of epilepsy, liver disease, renal disease, diabetes mellitus, alcoholism, gastrointestinal diseases/surgery, malabsorptive states or genetic disorders such as acrodermatitis enteropathica and sickle cell disease. Subjects were also excluded if they routinely used prescription/nonprescription medications; admitted to the use of recreational drugs or tobacco/tobacco products; had blood chemistry or hematological profiles that were not within the normal range; and/or were over/under-weight. Food records and questions related to adherence to special diets, food habits/beliefs/taboo, supplement use, fluctuations in body weight and concerns about body weight were used to select subjects for whom it was thought that compliance would be high.

#### Description of Subjects

Twelve adult (20-34 y) Caucasian males with an average (mean  $\pm$  SD) weight of  $71.3 \pm 10.6$  kg were selected. None of the subjects reported a history or current diagnosis of any of the medical conditions listed in the exclusion criteria. All subjects denied the use of prescription medications, recreational drugs and tobacco/tobacco products. Blood chemistry and hematological profiles were normal for all subjects, and review of the food records revealed that the subjects routinely consumed a typical nonvegetarian, Western diet.

### Experimental Design

The purposes, procedures, risks and benefits of the study were explained in detail to each of the subjects, and a copy of the informed consent form was provided to each of them for their perusal. Upon signing the informed consent, the subjects agreed to adhere to the study protocol which included: consuming all of, and only those foods and beverages provided by the researcher; avoiding oral contact with nonfood items; complying with urine and blood collection protocols and schedules; restricting use of personal hygiene products to those provided or approved by the investigator (Kauwell Methodology, 1993, Food Science and Human Nutrition Archives); conforming to the metabolic style of eating; completing a daily checklist (Kauwell Methodology, 1993, Food Science and Human Nutrition Archives); limiting exercise to one hour each day; abstaining from the use of nonprescription medications unless approved by the investigator; and informing the investigator of any changes in health status or unusual symptoms. Subjects were informed that if they were unable or unwilling to comply with any of these conditions they would be released from the study.

The study design was a single-blind crossover protocol as depicted in Figure 4-1. Subjects were fed a controlled constant diet for two, 28-day periods. No treatments were introduced during the first three days of each feeding period to allow the subjects time to adjust to the diet and to their

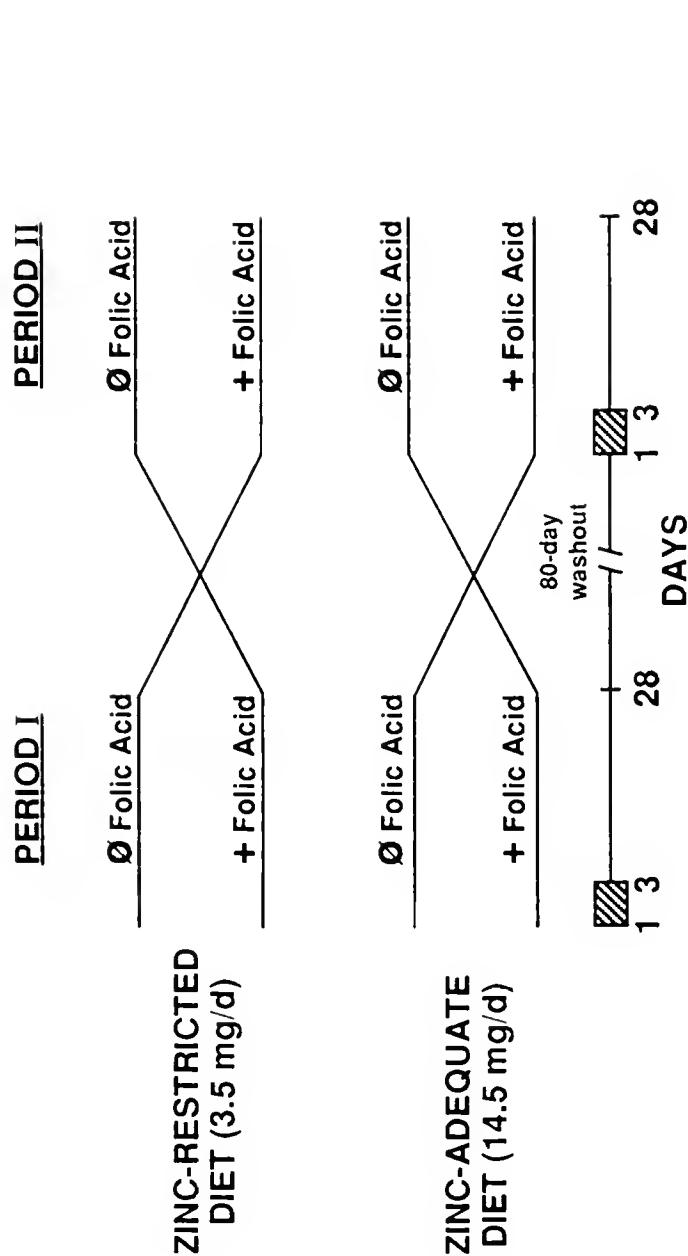


Figure 4-1. Experimental design.

new eating environment. A baseline 24-h urine collection was started after the first morning void on the third day of the study, and thereafter, collections were started on days 11, 18 and 27. Fasting blood samples were obtained on the morning of the fourth day of the study, and additional fasting blood samples were obtained on days 8, 11, 15, 21, 24 and 29. Beginning with breakfast on the fourth day of the study, the subjects were randomly assigned to receive a diet containing either 3.5 or 14.5 mg zinc/d. (Subjects assigned to the zinc-restricted or zinc-adequate diets were designated #1 through #6 and #7 through #12, respectively.) Half of the subjects in each zinc group were also randomly assigned to receive 800  $\mu$ g of deuterium-labeled folic acid monoglutamate administered daily in apple juice. The remaining subjects received plain apple juice served in an identical container. Adequacy of caloric intake was monitored by weighing subjects three times a week.

Upon completion of the first phase of the study, folic acid supplementation was discontinued and the subjects consumed self-selected diets for 80 days. The restrictions imposed on the subjects during the treatment phases of the study were lifted during the washout period; however, the subjects were not permitted to take vitamin/mineral supplements or engage in excessive alcohol consumption during this time. During the washout period, blood samples and five-day food records were obtained every three to four weeks. At

the end of the 80-day washout period, the study was repeated. During study period two, subjects consumed the same level of zinc to which they had been randomized during study period one; however, the folic acid/placebo treatments were switched so that subjects who did not receive supplemental folic acid during the first phase of the study received it during the second study period. The time intervals at which weights and blood and urine specimens were obtained were the same as those for study period one.

Blood samples were analyzed to determine the concentrations of plasma and erythrocyte zinc; serum and erythrocyte folate; erythrocyte metallothionein; serum alkaline phosphatase; and serum ferritin. The packed cell volume and the protein concentration of erythrocyte lysates were also determined. A complete blood count with differential, and a 25-item blood chemistry profile were performed by SmithKline Beecham Clinical Laboratories (Gainesville, FL) at three time points during each of the study periods. Twenty-four hour urine collections were analyzed for total urinary zinc and folate concentrations. The amounts of labeled and unlabeled folate excreted in the urine were also quantified. Aliquots of meal composites prepared on two separate occasions during both study periods were analyzed to determine the total zinc content of the diet. Food records collected during the prestudy and washout periods were evaluated by computer (Practorcare 4000, San Diego, CA)

to determine the average intake of calories, protein, folate and zinc from self-selected diets.

### Materials and Methods

#### Description of Diet and Supplements

A three-day cycle menu containing a daily average of 2985 kilocalories (42 kcal/kg body weight), 80 g protein (1.1 g/kg body weight), 290  $\mu$ g folate, and by actual analysis, 3.5 mg zinc, was developed for this study (Appendix B). The three-day average nutrient content for other nutrients, as determined by computer analysis (Practorcare 4000, San Diego, CA), is listed in Table 4-1. In addition to the diet, supplements were provided so that the experimental diet would more closely approximate the 1989 RDA for all nutrients. Consistency in nutrient content for repeated days of the diet was maintained by purchasing foods according to case lot number. The menu was the same for both study periods and consisted of conventional foods fed as three meals and one snack. In order to provide enough protein in the diet, the subjects also received two "protein shakes" per day (Table 4-2). The diet consumed by subjects in the zinc-adequate group was identical to that consumed by the zinc-restricted group except that 5.5 mg zinc, as zinc sulfate (VWR Scientific, Atlanta, GA), was added to each protein shake to provide a total zinc intake of 14.5 mg/d.

Table 4-1. Average nutrient content of three-day diet with and without supplements.

<u>Nutrient</u>	<u>Diet alone</u>	<u>Supplements alone</u>	<u>Diet+ supplement</u>
kilocalories	2,985	same	same
protein, g	80	same	same
carbohydrate, g	465	same	same
fat, g	90	same	same
vitamin A, IU	12,387	same	same
vitamin C, mg	420	same	same
thiamin, mg	1.2	0.7	1.9
riboflavin, mg	2.0	0.7	2.7
niacin, mg	21	9	30
vitamin B <sub>6</sub> , mg	1.7	0.9	2.6
pantothenic acid, mg	2.8	same	same
folate, µg	290	*	*
vitamin B <sub>12</sub> , µg	0.9	2.0	2.9
calcium, mg	482	200	682
phosphorous, mg	869	same	same
sodium, mg	3,363	same	same
potassium, mg	2,564	same	same
iron, mg	12	same	same
magnesium, mg	165	81	246
zinc, mg	3.5	*	*
copper, mg	0.9	2.0	2.9
selenium, µg	56	same	same
biotin, µg		300	300

\*Zinc and folic acid supplementation provided as per the experimental design and randomization scheme.



Table 4-2. Composition of protein shakes.

<u>Ingredients</u>	<u>Amount per serving</u>
powdered, sweetened, instant drink mix	67.5 g
egg whites, (frozen, pasteurized)	150.0 g
frozen nondairy creamer	52.5 g
doubly deionized, distilled water	<u>90.0 g</u>
	360.0 g

All meals were prepared in the University of Florida, College of Medicine, Clinical Research Center Metabolic Kitchen. Standardized recipes, food preparation techniques and food service and sanitation procedures (Kauwell Methodology, 1993, Food Science and Human Nutrition Archives) were developed and followed to ensure uniformity. Doubly deionized, distilled water, hereafter referred to as water, was used during all aspects of food preparation, service and sanitation. Other procedures followed in order to avoid trace mineral contamination are discussed in a succeeding subsection.

With the exception of ready-to-consume foods available in prepackaged individual portion sizes (i.e. potato chips, corn chips, fruit juices, salad dressings and breakfast cereals), all foods and ingredients used in meal preparation were weighed before they were served to the subjects. During the first three days of the study (i.e. prior to initiation of the

treatments), subjects were given the opportunity to decide if they wished to include up to one gram of salt and/or one package of instant coffee reconstituted with water. Once the subjects committed to either or both of these options, they were required to consume these foods daily for the duration of both treatment periods. Subjects were also offered the option of including up to 32 oz of Diet 7<sup>UP</sup>® (Pepsico, Inc., Somers, NY), three sticks of spearmint or peppermint Carefree® Sugarless Gum (Planter Lifesavers Co., Winston Salem, NC), and/or one package of Equal® (NutraSweet Co., Deerfield, IL) on a daily basis. Water was provided to all subjects ad libitum.

Subjects consumed breakfast and dinner at the same time every day in the Clinical Research Center. Breakfast and dinner trays were checked for accuracy prior to meal service to avoid omission of any foods/beverages and to ensure that each subject received the appropriate treatments. Subjects consumed their meals using the eating techniques in which they had been instructed, which included using a rubber spatula and a rinse bottle to loosen any food particles or residues remaining on their dishes or beverage containers. Bread, which was served at both meals, was used to absorb any liquid remaining on the serving dish or container. At the end of each meal, the subjects' trays were checked for completeness of consumption. A bag lunch and an evening snack were distributed to subjects after they completed their breakfast

and dinner meals, respectively. Subjects consumed this meal and snack on their own at noon and 10:00 pm, respectively, using the eating techniques in which they had been instructed. No treatments (i.e. additional zinc or deuterium-labeled folic acid) were administered at lunch or as part of the evening snack.

The supplements provided to the subjects included calcium citrate (Citracal 950®, Mission Pharmacal Co., San Antonio, TX), magnesium gluconate (Willner Chemists, Inc., New York, NY) and vitamin B complex (Squibb®, Princeton, NJ) in tablet form. Copper, as cupric sulfate (VWR Scientific, Atlanta, GA), was dispensed into the protein shakes. Biotin tablets (Puritan's Pride®, Bohemia, NY) were provided as a safeguard to protect against the possible development of a biotin deficiency due to the use of egg whites in the protein shakes. All supplements were consumed in the presence of the investigator.

Supplemental folic acid was provided as 3',5' deuterium-labeled folic acid monoglutamate. This stable isotope of folic acid was synthesized by Dr. J.F. Gregory, III, using an improved labeling procedure that yields complete labeling of the 3' and 5' positions of the folic acid molecule (Gregory, 1990). In this procedure, unlabeled folic acid is brominated at the 3',5'-positions, followed by catalytic debromination with deuterium gas and a palladium/carbon catalyst. The labeled folic acid is purified chromatographically, and the

degree of purity is established using analytical HPLC. Finally, the site and degree of deuterium labeling is confirmed by mass spectrometry and proton nuclear magnetic resonance.

To prepare the supplement for consumption by the subjects, the amount of deuterium-labeled folic acid needed to make enough 800  $\mu$ g dose portions for the first study period was weighed; dissolved in a small amount of 1.0 M sodium hydroxide (NaOH); diluted with 0.1 M phosphate-buffered saline (PBS) (pH 7.0); adjusted to pH 7.0 with HCl; and brought to volume with more buffer. The volume of the deuterium-labeled folic acid solution to be dispensed into each tube of apple juice was calculated after determining the folic acid concentration spectrophotometrically at 280 nm. The labeled folic acid was dispensed into 50 mL conical centrifuge tubes (Corning®, Corning, NY) containing 45 mL of apple juice. The tubes were flushed with nitrogen, sealed and stored at -20° C. Precautions were taken during all phases of preparation, storage and service to protect the folic acid from light. Plain apple juice served in identical containers was used as the placebo.

#### Procedures Used to Foster, Monitor and Assess Compliance

Compliance was fostered by maintaining close personal interaction with the subjects and reinforcing the importance of adherence to all aspects of the protocol. Subjects were

also asked to sign contracts (Appendix C) that detailed their responsibilities. Methods used to monitor and assess compliance to the protocol included: 1) observing the subjects during meal times and checking their trays to make sure that all foods and beverages were consumed; 2) completion and signing of a daily checklist addressing compliance issues (Kauwell Methodology, 1993, Food Science and Human Nutrition Archives); and 3) completion of an anonymous questionnaire at the end of the study to identify any deviations from the protocol.

#### Procedure Used to Assess Adequacy of Blinding

In order to determine if subjects had been adequately blinded to the level and order of treatments they received, subjects were asked to complete a questionnaire addressing these issues at the end of the study. The questionnaire listed the possible combinations of treatments the subjects could have received during each of the study periods and asked them to identify which combination of treatments they thought they had received.

#### Procedures Used to Prevent Zinc Contamination

Hair restraints and powder-free gloves were worn during all aspects of food handling, preparation and sanitation in order to prevent incidental zinc contamination. All work surfaces and food service areas were sanitized with a diluted

solution of bleach, after which they were rinsed with water. The lids of canned foods were treated in the same manner before they were opened to prevent incidental zinc contamination.

Samples of all disposable containers and utensils used for food preparation and/or service were tested for zinc contamination before the study was begun. Cups, dishes, bowls, plastic utensils, polypropylene conical centrifuge tubes (Corning®, Corning, NY), plastic bags and other disposable items were filled/soaked in 0.1 N hydrochloric acid (HCl) overnight. Duplicate samples of HCl from each disposable item were analyzed for the presence of zinc by air/acetylene flame atomic absorption spectrophotometry (AAS) (Perkin-Elmer® 2600, Norwalk, CT). Only containers/utensils determined to be zinc-free were used. Vials and containers used to collect and/or store blood or urine samples to be analyzed for their zinc content were tested in the same manner.

Food service equipment, containers and utensils to be used repeatedly throughout the study were also tested for zinc contamination. Since it was important to know if the procedures to be used for sanitizing these items would be a source of incidental zinc contamination, all items were sanitized before they were tested. The sanitation process included washing the items in a household automatic dishwasher with a prescribed amount of dish washing detergent, rinsing

them three times with water (manually, while wearing powder-free gloves) and allowing them to air dry. Gallon and half-gallon plastic beverage containers used to supply the subjects with drinking water, were sanitized manually since the dishwasher would not accommodate these items. These containers were also rinsed three times with water and air dried. Only equipment, containers and utensils for which there was no evidence of zinc contamination were used in the study. Every time these items were used, they were cleaned using the same procedures.

The protein shakes consumed by the subjects were served in sixteen ounce, reusable, plastic drinking cups with lids (Packard Plastics, Lawrence, KS). Since zinc sulfate was added to some of the shakes, the cups and lids were washed in an automatic dishwasher and then submerged in a solution of Radiacwash® (Atomic Products Corporation, Shirley, NY), a metal-chelating agent, in order to prevent incidental zinc contamination. After soaking in the Radiacwash® solution, the cups and lids were vigorously rinsed six times (powder-free gloves were worn) and then air dried. Containers used for collecting urine were processed in a similar manner except that they were soaked in bleach before being washed by hand. Separate soaking tubs were used for drinking cups and urine containers.

### Urine Collection and Processing Procedures

Opaque urine containers with capacities of 2.5 L and 0.5 L were cleaned in the manner described above. Free ascorbate (1.5 g/L) was added to each container to protect urinary folates. Subjects were provided with written and verbal instructions on how to collect their urine. Urine was collected after the first void of the day for a period of 24 h. Subjects were instructed to void directly into the container, but to avoid touching the inside of the container; to completely fill one container before starting a new container; to keep the urine they collected cool at all times; and to return all containers to the investigator at the end of the 24 h collection period.

Precautions were taken to avoid zinc contamination while processing the urine. The volume of the urine excreted by each subject was measured using acid-washed graduated cylinders. Powder-free gloves were worn during the processing operation, and samples were stored in zinc-free containers. Aliquots of well-mixed urine, to be used to determine the concentrations of urinary zinc, urinary folate and deuterium-labeled folates, were protected from light and stored at -20° C.

### Blood Collection and Processing Procedures

Fasting morning blood samples were drawn by a trained phlebotomist. A description of the equipment, supplies and



procedures used to obtain, collect and process the specimens is outlined below. Doubly deionized, distilled water, hereafter referred to as water, was used exclusively for all aspects of sample processing and analysis.

Plasma/erythrocyte zinc and erythrocyte metallothionein.

Precautions were taken to avoid zinc contamination during sample collection and processing. All equipment, solutions and vials used for blood collection and processing were tested and found to be zinc-free. A blood collection set with multiple sample luer adapter (Vacutainer®, Becton Dickinson, Rutherford, NJ) was used in combination with two, 10 mL polypropylene syringes (Sarstedt, Princeton, NJ) to draw blood for determination of plasma zinc, erythrocyte zinc and erythrocyte metallothionein concentrations. Sodium heparin (Lyphomed, Inc., Rosemont, IL) was added to each syringe before the blood samples were drawn. Samples were held on ice, and processing was begun within one hour after the samples were obtained.

Duplicate samples were centrifuged in the original container at 1500g for 12 minutes at 4° C. The plasma fraction was separated from each sample and stored at -20° C. The remainder of each blood specimen was used to prepare duplicate samples of erythrocyte lysates. The buffy coat was removed from the erythrocyte pellet, and an equal volume of ice-cold 0.9% sodium chloride (NaCl) was added. The samples were centrifuged for 5 minutes, after which the supernatant

was removed from the pellet. This process was repeated two times. After the final wash, the supernatant was removed and discarded, and 2.5 mL of packed red blood cells were mixed with 1 mL of ice cold water. The samples were frozen at  $-70^{\circ}\text{C}$ .

Serum/erythrocyte folate, serum ferritin and serum alkaline phosphatase. Precautions were taken to protect samples from light during collection, processing and storage. Samples used to determine serum concentrations of analytes were collected in 13 mL silica coated tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ) and held at room temperature for 30 to 60 minutes to allow time for clotting. The clotted portion of each sample was removed, after which the samples were centrifuged. The serum samples were removed, placed in clean centrifuge tubes and centrifuged again before preparing separate aliquots of the serum to be analyzed for serum ferritin and serum folate concentrations. Ascorbic acid (1 mg/mL) was added to vials to be analyzed for serum folate to ensure stability during storage. All serum samples were stored at  $-20^{\circ}\text{C}$ . On days 4, 15 and 29 of the study, serum samples were dispensed into a third set of vials and sent to a clinical laboratory (SmithKline Beecham Clinical Laboratories, Gainesville, FL) for routine laboratory tests. Serum alkaline phosphatase activity was determined as a component of this routine blood chemistry profile using the method of Bowers and McComb (1966; 1975).

Samples for whole blood analysis were collected in 10 mL ethylenediamine tetraacetic acid (EDTA) tubes (Vacutainer®, Becton Dickinson, Rutherford, NJ). Prior to processing, duplicate samples of blood were drawn into capillary tubes and centrifuged for 5 minutes at approximately 12,000 rpm. Hematocrit levels were determined using a microhematocrit tube reading device. The remaining sample was diluted 1:10 with 0.1 % sodium ascorbate and allowed to incubate at room temperature for 30 minutes. After incubation, PBS (0.2 M) was added to a final dilution of 1:20, and this whole blood lysate was stored at -20° C.

#### Preparation of Diet Composites

All foods and beverages served to the subjects on a given day were assembled. Foods that required cooking were prepared according to the procedures established for this study. A rinse bottle was filled with hot water, and the water was sprayed around the edges of dishes containing cooked foods as soon as they were removed from the oven to prevent the food from sticking to the cooking dish. Solid foods were transferred to a stainless steel blender, before transferring liquids, to avoid the loss of liquids due to splashing. Crumbs remaining in the original serving container were loosened with a spatula and subsequently emptied into the blender. Beverages were poured into the blender, and the serving carton/cup was rinsed with water and scraped dry with

a spatula. A portion of the daily bread allowance was used to wipe any liquid remaining in the serving containers. Bread was also used to wipe residues left in the serving containers from oil-based foods such as salad dressing and mayonnaise. After all foods and beverages had been added to the blender, the spatula was rinsed over the top of the blender and dried with a small piece of bread. The diet was blended on low speed for two minutes and high speed for two minutes.

A preweighed, gallon size, grip lock plastic bag was tared on a scale and half of the blended diet was poured into it. The remaining blended diet was poured into a second bag. The weights of both bags were recorded. The bags were sealed and placed flat on a tray in a -4° C freezer. After the bags were frozen solid, they were freeze-dried. The bags of freeze-dried diet were reweighed to determine the dry weights of the diets for each day of the cycle menu. Each day of the menu was prepared in duplicate during both study periods. Powder-free gloves were worn throughout the entire process to prevent zinc contamination.

#### Determination of Dietary Zinc Content

Unused, 100 mL high-form porcelain crucibles were soaked in 10% nitric acid, dried in an oven at 105° C overnight and transferred to desiccators containing silica dioxide. After cooling, approximately 6 g of freeze-dried diet were added to each labeled crucible. The exact weight of the empty crucible

and the weight with the sample were noted. Triplicate samples were prepared from each bag of freeze-dried diet, resulting in a total of six samples for each day of the cycle menu. The crucibles containing the samples, and an empty crucible to be used as a blank, were then returned to the 105° C oven for overnight drying. In the morning, crucibles were removed from the drying oven, placed in desiccators to cool, reweighed and transferred to a muffle furnace with an initial temperature of 200° F. The temperature was increased by 100° F every hour until the internal oven temperature reached 550° F. This temperature was held overnight and cooled to 200° F in the morning, after which, the crucibles were transferred to desiccators for cooling.

After cooling, the ashed samples were dampened with water and transferred to a prewarmed hot plate. Approximately 10 mL of 50% HCl were added to each crucible. The liquid was evaporated to half volume and then the crucibles were filled to two-thirds of their capacity with 10% HCl. The HCl was evaporated to a level representing approximately 10 mL, after which the crucibles were filled to two-thirds of their capacity with water. This was evaporated to about 5 mL of liquid, at which time the samples were removed from the hot plate for cooling.

Each solubilized sample was filtered into a labeled 25 mL volumetric flask using an extended neck glass funnel and 11 cm Whatman filter paper. After filtering, a plastic paddle and

water were used to loosen and solubilize residues that had accumulated on the internal surfaces of the crucibles. The rinse water was filtered, and this process was repeated two more times. (All glassware and paddles were acid washed in 10% nitric acid and thoroughly rinsed with water before they were used.) When the funnels ran dry, the filter papers and the funnels were rinsed with a small amount of water. Each flask was brought up to volume with water. The flasks were covered, inverted and mixed by agitation. The samples were transferred into labeled zinc/trace element-free polypropylene tubes (Sarstedt, Princeton, NJ).

Standards were made in a matrix containing calcium, magnesium, phosphorous, potassium and sodium in proportions similar to those in the diet (as determined by computer analysis). The matrix solution also contained 250 mL of 10% HCl and water. The matrix was added to 100 mL volumetric flasks containing 1, 3 and 6 mL, respectively, of 100 ppm zinc standard prepared from 1000 ppm Certified Zinc Reference Solution (Fisher Scientific Co., Pittsburgh, PA). These solutions were mixed well and poured into labeled zinc/trace element-free polypropylene tubes. The National Institute of Standards and Technology's standard reference material #1572 (i.e. citrus leaves) was dried and dissolved following the manufacturer's directions.

The absorbance readings of the blank, standards and samples were determined by air/acetylene flame AAS using a

Perkin-Elmer® 5000 atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT) set at 213.9 nm. Linear regression analysis was used to calculate the average zinc concentration for the reference standard and each day of the cycle menu.

### Biochemical Analyses

#### Plasma and Urine Zinc Concentrations

Plasma zinc concentrations were measured by air/acetylene flame AAS at a wavelength of 213.9 nm, using a Perkin-Elmer® 2380 (Perkin-Elmer Corporation, Norwalk, CT) flame atomic absorption spectrophotometer. Duplicate plasma samples were diluted five-fold with water. Metal-free pipette tips and metal-free 1.5 mL polypropylene test tubes (Biorad, Richmond, CA) were used to prepare these samples. Pooled samples were prepared in the same manner. A sample of the National Institute of Standards and Technology's standard reference material #1598 (bovine serum) was prepared for recovery to evaluate the accuracy of the analytical method. Certified Zinc Reference Solution (1000 ppm; Fisher Scientific Co., Pittsburgh, PA) was used to make the zinc standards. These standards were prepared in duplicate in 5% glycerol to minimize differences due to viscosity (Smith and Butrimovitz, 1979). A solution of 5% glycerol was used for the blank. All glassware used to prepare the standards and blank had been acid-washed (10% nitric acid solution) and thoroughly rinsed

in water. Each sample/standard was vortexed just before aspiration. A pooled sample was aspirated every fifth sample and the standards were aspirated every tenth sample. Hydrochloric acid (0.1 N), followed by water were aspirated between each sample/standard. Sample concentrations were calculated using linear regression from the standard concentration line.

Duplicate urine samples were vortexed and aspirated directly using the same spectrophotometer used for the plasma samples. Standards for urine zinc were prepared in 0.1 N HCl. As a control, lyophilized Gilford Urine Metal Control II (Ciba Corning Diagnostics Corporation, Irvine CA) was dissolved in water and diluted with 0.1 N HCl. Standards and the control were checked every tenth sample. Sample concentrations were calculated using linear regression from the standard concentration line.

#### Erythrocyte Lysate Zinc Concentration

The zinc concentrations of erythrocyte lysates were measured by air/acetylene flame AAS at a wavelength of 213.9 nm, using a Perkin-Elmer® 5000 (Perkin-Elmer Corporation, Norwalk, CT) flame atomic absorption spectrophotometer. Duplicate erythrocyte lysate samples were diluted to a fourteen-fold final dilution with water. Metal-free vials and pipette tips (Biorad, Richmond, CA) were used. Pooled samples were prepared in the same manner. Certified Zinc Reference



Solution (1000 ppm; Fisher Scientific Co., Pittsburgh, PA) was used to make the zinc standards. Zinc standards were prepared in duplicate in 5% glycerol. A solution of 5% glycerol was used for the blank. All glassware used to prepare the standards and blank had been acid-washed (10% nitric acid solution) and thoroughly rinsed in water. Each sample/standard was vortexed just before aspiration. A pooled sample was aspirated every seventh sample and the standards were aspirated every fifteenth sample. Hydrochloric acid (0.1 N), followed by water were aspirated between each sample/standard. Sample concentrations were calculated using linear regression from the standard concentration line. The final zinc concentrations were expressed per milliliter of erythrocyte lysate and per gram of protein.

#### Protein Determination

Protein concentrations of erythrocyte lysates were determined by the Folin phenol reagent method of Lowry et al. (1951). One milliliter of Lowry Reagent (0.58 mM  $\text{Na}_2\text{CuEDTA}$ , 0.18 M  $\text{Na}_2\text{CO}_3$  and 0.1 M NaOH) was added to duplicate samples diluted 1:700 and to standards prepared using bovine serum albumin (BSA) (Sigma® Chemical Co., St. Louis, MO). Samples and standards were vortexed and incubated for 10 minutes, after which 0.1 mL of phenol reagent (Folin-Ciocalteu Phenol Reagent, Sigma® Chemical Co., St. Louis, MO) was added. After 30 minutes, absorbance was read at 500 nm (Beckman DU-64

Spectrophotometer, Palo Alto, CA). Sample concentrations were calculated using linear regression from the standard concentration line.

#### Erythrocyte Metallothionein Concentration

Metallothionein (MT) concentrations of erythrocyte lysates were measured using an ELISA for human metallothionein-1 (Grider et al., 1989). In this assay, primary antibody (i.e. sheep anti-human metallothionein-1 immunoglobulin G) directed against human metallothionein-1 binds in a competitive fashion to human metallothionein-1 coated to the wells of a microtiter plate and to free metallothionein present in the sample or standard. The antigen-antibody complex formed between the primary antibody and the free metallothionein in the sample/standard is removed, leaving behind only the primary antibody bound to the human metallothionein coated to the wells of the plate. Enzyme-linked secondary antibody (donkey anti-sheep IgG/alkaline phosphatase conjugate, Sigma® Chemical Company, St. Louis, MO) is added to the plate, and this binds with the antigen-antibody complex. Enzyme-linked secondary antibody that does not bind to the antigen-antibody complex is removed. Following incubation with para-nitrophenyl phosphate, color development occurs, and absorbance is measured spectrophotometrically at 405 nm. Metallothionein-1 concentrations of unknown samples are determined by linear

regression of the standard curve after logit Y transformation. A schematic presentation of this assay is presented in Figure 4-2.

Purification of human metallothionein. Human metallothionein-1 used for the coating antigen and standard were provided by Dr. R.J. Cousins. Pooled human liver samples obtained from the College of Medicine at the University of Florida, Gainesville Florida, were homogenized and centrifuged. The supernatant was subjected to gel filtration chromatography, and the zinc-containing fractions that comprised the metallothionein peak were fractionated by anion exchange HPLC. The metallothionein isoforms were separated using a step gradient generated by a gradient pump. All metallothionein-1 fractions were pooled and concentrated by ultrafiltration. The purity of metallothionein-1 was determined by amino acid analysis (Grider et al., 1989).

Production of sheep anti-human metallothionein-1 immunoglobulin G. Sheep anti-human metallothionein-1 immunoglobulin G (primary antibody) was provided by Dr. R.J. Cousins. Purified human metallothionein-1 was incubated overnight with purified rat immunoglobulin G (Sigma® Chemical Co., St. Louis) in PBS. Prior to injection, this solution was combined with an equal amount of a 1:1 mixture of Freund's complete/incomplete adjuvant. Sheep received multiple intradermal and intramuscular injections of this solution, with booster injections given at 30, 50 and 106 days following

the initial injection. Serum was harvested seven days after each booster injection. The sheep anti-serum was applied to a protein A agarose column (Bethesda Research Laboratories, Gaithersburg, Md.), and fractions containing peak absorbance at 280 nm (anti-human metallothionein-1 immunoglobulin G) were pooled and assayed for protein concentration (Grider et al., 1989).

Preparation of standard, pooled control and samples.

Purified human metallothionein-1 and a pooled erythrocyte lysate with known concentrations of metallothionein-1 were provided by Dr. R.J. Cousins. The purified human metallothionein-1 was diluted 1:20 with a solution of 10 mmol/L PBS, 1% BSA, 0.5% polyoxyethylene sorbitan monolaureate (i.e. Tween 20) (Sigma® Chemical Company, St. Louis, MO) and 0.02% sodium azide ( $\text{NaN}_3$ ), pH 7.2. The unknown samples were diluted 1:1 with the same solution to which 2-mercaptoethanol was added at a level of 2  $\mu\text{L}/\text{mL}$  of diluent.

ELISA procedure. Coating antigen (1 mL human metallothionein-1 antigen, 100 ng/mL) was added to 9.5 mL of PBS (10 mmol/L, pH 7.2) and 10  $\mu\text{L}$  of 2-mercaptoethanol. Aliquots (100  $\mu\text{L}$ ) of this solution were dispensed into the wells of a microtiter plate (NUNC, USA Scientific, Ocala, FL). The plate was covered and stored at 4° C for 16 h, after which the coating solution was discarded. The plate was washed three times with a washing solution consisting of 10 mmol/L PBS, plus 0.5% Tween 20. To reduce nonspecific binding,

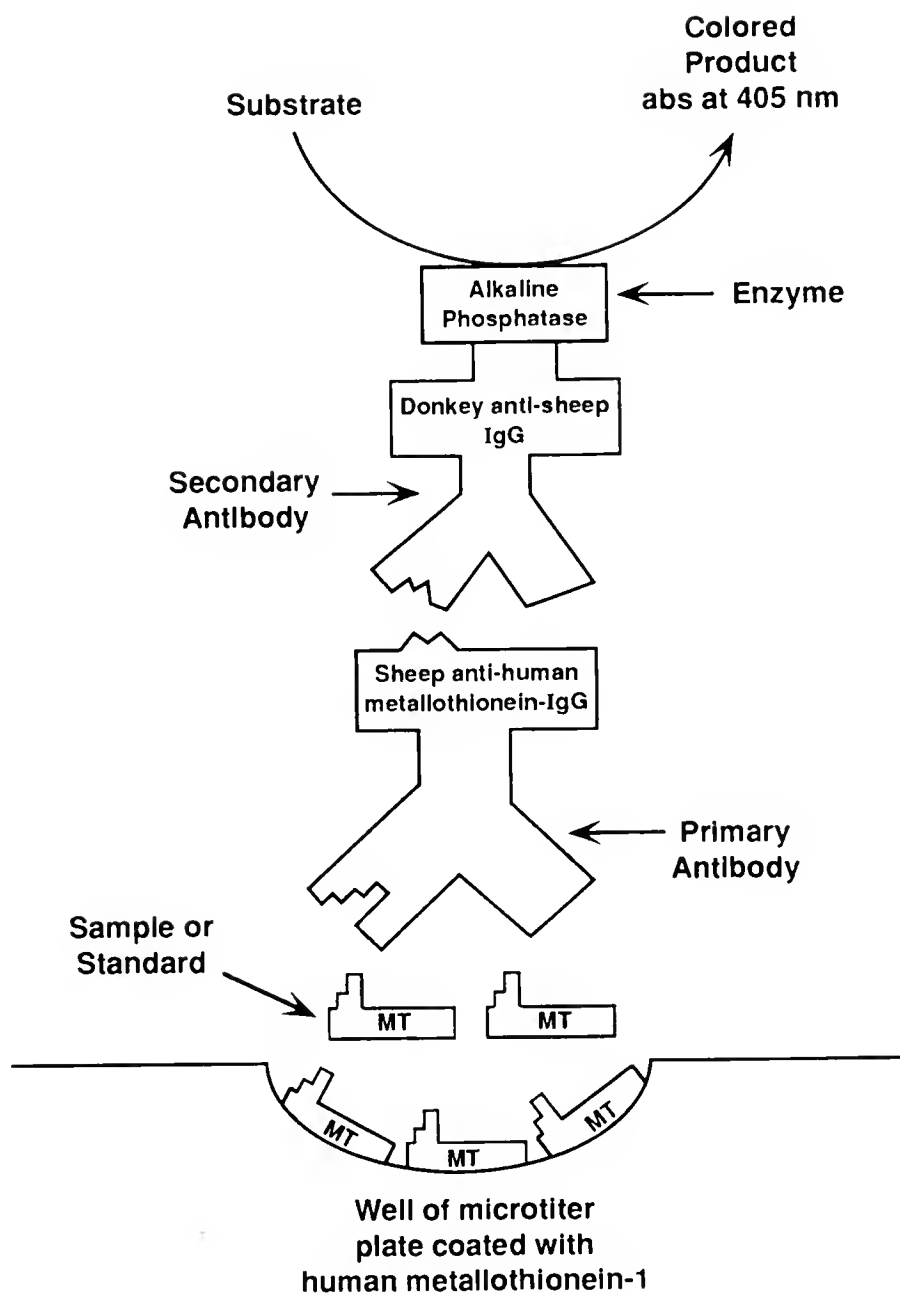


Figure 4-2. Schematic of enzyme-linked immunosorbent assay used for determination of erythrocyte metallothionein concentrations.

300  $\mu\text{L}$  of buffer solution (10 mmol/L PBS, 1% BSA, 0.5% Tween 20, 0.02%  $\text{NaN}_3$ , pH 7.2) were added to all wells of the plate, and the plate was incubated at room temperature for 30 minutes. The buffer solution was discarded and the plate was washed with the washing solution and tapped dry. Fifty microliters of the buffer solution, and 100  $\mu\text{L}$  of the standard, pooled control and samples were added to designated wells of the plate. The samples, pooled control and standard were added in duplicate. A multi-channel pipette was used to perform a total of four serial dilutions, after which, buffer-diluted primary antibody (i.e. 3  $\mu\text{L}$  antibody plus 10.5 mL buffer) was dispensed (50  $\mu\text{L}$ /well) into the appropriate wells. The plate was covered and incubated at room temperature for 4 h. The solution in the wells of the plate was discarded at the end of the incubation period, and the plate was washed and tapped dry. Buffer-diluted secondary antibody (20  $\mu\text{L}$  of secondary antibody plus 10.5 mL buffer) was added (100  $\mu\text{L}$ /well) to all wells of the plate and incubated for 30 minutes at room temperature. The solution was discarded again, and the plate was washed and tapped dry. A substrate solution of para-nitrophenyl phosphate (i.e. 4 mol/L in carbonate buffer; one tablet of para-nitrophenyl phosphate disodium mixed with 41 mL of carbonate buffer, pH 9.6) was added to all wells of the plate (200  $\mu\text{L}$ /well) and incubated in the dark at 37° C for 1 h. Absorbance at 405 nm was measured using a microtiter plate reader (Molecular Devices Corporation

UV Max, Menlo Park, CA) interfaced with a computer. Metallothionein concentrations of unknown samples were determined by linear regression of the standard curve after logit Y transformation. The final results were expressed as  $\mu\text{g/g}$  protein.

#### Determination of Serum Ferritin Concentration

Serum ferritin concentrations were determined using a sandwich-type ELISA (Flowers et al., 1986). Rabbit, anti-human ferritin (Dako Corporation, Carpinteria, CA) diluted 1:500 in 0.1 M bicarbonate buffer (pH 9.6) was used to coat the wells of a 96-well microtiter plate (NUNC, USA Scientific, Ocala, FL). The plate was covered and incubated at room temperature for 6 h, after which the coating solution was discarded and the plate was washed three times with a solution of 0.05% Tween 20/Dulbecco's phosphate-buffered saline (DPBS). Ferritin standard (10  $\mu\text{g/mL}$  in NaCl; ICN Biochemicals, Cleveland, OH), diluted with 1% BSA/DPBS, was added to the plate and serially diluted with additional 1% BSA/DPBS. The samples, diluted 1:5 with the same solution as the standards, were added to the plate in triplicate. The plate was covered, refrigerated overnight and then washed with 1%BSA/DPBS. Horseradish peroxidase conjugated ferritin (Dako Corporation, Carpinteria CA), diluted with 1%BSA/DPBS to a final concentration of 1:4000, was added to the plate. The plate was covered, incubated for 6 h at room temperature and then

washed. A substrate solution consisting of 1,2-orthophenylenediamine, dihydrochloride (Dakopatts, Denmark) dissolved in 0.1 M citric acid-phosphate buffer with 30% hydrogen peroxide (HRP Color Reagent B, BioRad Laboratories, Richmond, CA) was added to the plate, and the plate was placed in the dark for 15 minutes to allow for color development. Absorbance was read at 490 nm using a Molecular Devices Corporation UV Max microtiter plate reader (Menlo Park, CA). Sample concentrations were calculated using linear regression from the standard concentration line.

#### Determination of Serum, Whole Blood and Urinary Folate Concentrations

Total folate concentrations of serum, whole blood and urine samples were determined microbiologically using the 96-well microtiter plate assay (Newman and Tsai, 1986). The assay organism for the microbiological assay was *Lactobacillus casei* (*L. casei*) grown in ATCC 7469 (Difco Laboratories, Detroit, MI) growth media. Preparation of the standard, samples and culture organism, as well as performance of the assays, were done under controlled lighting.

Preparation of media and reagents. Dehydrated folic acid casei medium (Difco Laboratories, Detroit, MI), was reconstituted, filter sterilized using a Corning® filter/storage system with a 0.22 micron cellulose acetate filter (Corning Glass, Corning, NY) and stored at 4° C. The same type of filtration system was used to sterilize a 0.1 M



phosphate buffer solution (pH 6.3) containing 1 mg/mL ascorbic acid, which was prepared immediately before each assay was begun.

Microorganism. Freeze-dried cultures of *L. casei* 7469 were obtained from American Type Culture Collection (Rockville, MD). Dry culture was suspended in a sterile solution containing 25 mL of media and an equal volume of 0.1 M phosphate buffer solution (pH 6.3) containing 1 mg/mL ascorbic acid. Folinic acid (Sigma® Chemical Company, St. Louis, MO; 1 ng/mL) was added to the suspended culture, and the culture was incubated at 37° C for 24 h.

After demonstrating that the assay organism provided an adequate folate response curve, the cryoprotected assay organism was prepared. Approximately, 0.5 mL of the previous day's growth was added to a 200 mL sterile receptacle (Corning Glass, Corning, NY) containing 25 mL of sterile medium, 25 mL of 0.1 M potassium phosphate buffer (pH 6.3), 50 ng folinic acid and 50 mL of sterile 80% glycerol. The solution was mixed well and 0.5 mL aliquots were pipetted into sterile vials (Sarstedt, Princeton, NJ). The vials were stored at -20° C. Prior to inoculating the samples and standards, an aliquot of the cryoprotected microorganism was allowed to come to room temperature, and for each plate to be assayed, 45  $\mu$ L of the microorganism were diluted with 15 mL of sterile medium.

Preparation of standard. Folinic acid (150 mg; Sigma® Chemical Company, St. Louis, MO) was added to a 50 mL sterile conical centrifuge tube (Corning Glass, Corning, NY) and dissolved in sterile water (15 mL) by adding 0.1 N NaOH. The pH was adjusted to 7.0 with 0.1 N HCl, and the volume was adjusted to 25 mL with sterile water. After filtration, a 1:100 dilution of the standard was made. One milliliter of this standard solution was diluted with 2 mL 2N NaOH, and the absorbance at 282 nm was determined using a Gilford 250 spectrophotometer (Ciba Corning Gilford Systems, Oberlin, OH). The concentration of the standard was calculated using an extinction coefficient of 28,200. Aliquots of the standard (1:100 dilution) were stored at -20° C. Prior to performing the assay, the standard was defrosted and diluted with the ascorbate-phosphate buffer (pH 6.3) described above. The concentration of the folate standard used to construct the standard curve ranged from 0.023 to 0.375 pmoles/well.

Assay procedure using 96-well microtiter plate. Falcon® (Becton Dickinson, Rutherford, NJ), 96-well, flat bottomed, sterile, low evaporation tissue culture plates with lids (Becton Dickinson, Lincoln Park, NJ) were used for this assay. The assay was performed under a laminar flow hood using sterile equipment, supplies and solutions.

For each assay, water was added (300 µL) to the wells of the plate that were designated as blanks. One hundred and thirty microliters of the ascorbate-phosphate buffer and 20 µL

of appropriately diluted standard, pooled controls and samples were added in duplicate to selected wells of the plate. (Whole blood samples were diluted with buffer to a final concentration of 1:30 or 1:40, and urine samples were diluted 1:2, 1:4 or 1:8 before being added to the plate; serum samples were not diluted prior to being added to the plate.) One hundred and fifty microliters of buffer were dispensed into all other wells of the plate. A total of five serial dilutions of the samples, standard and pooled control were made using a multi-channel pipette. All wells were inoculated with 150  $\mu$ L of the medium containing the assay organism, and the plates were covered and placed in an incubator at 37° C for 16 h.

After incubation, the contents of each well were resuspended by repeated aspiration and flushing using a multi-channel pipette. A flame was quickly passed over the surface of the plate to eliminate air bubbles. Growth of the microorganism was measured by reading the turbidity of each well at 650 nm using a microtiter plate reader (Molecular Devices UV Max, Menlo Park, CA) interfaced with a computer. The output generated was used to calculate the folate concentration of the original serum, whole blood or urine sample. Once serum and whole blood folate concentrations were

determined, erythrocyte folate concentrations were calculated using the following formula:

$$\frac{[\text{whole blood folate}] - [\text{serum folate} \times (1 - \text{hematocrit}/100)]}{\text{hematocrit}} \times 100$$

#### Determination of Urinary Deuterium-Labeled Folate

Urinary excretion of deuterium-labeled folate (D<sub>2</sub>-FA) was determined using the method of Gregory and Toth (1988). This method involves the separation of folates using affinity chromatography, chemical cleavage of the folates to para-aminobenzoyl glutamate (pABG), purification of the pABG fragments using HPLC and quantification by GCMS.

Affinity chromatography column preparation. The columns used for affinity chromatography were provided by Dr. J.F. Gregory, III. These 2 mL columns were packed with Affigel-10® (Biorad, Richmond, CA), and folate binding protein isolated from bovine whey was bound to the Affigel-10®. Five milliliters of 0.1 N HCl were added to each column to remove any residual folates, and each column was equilibrated with 15 mL of 1.0 M potassium phosphate (pH 7.0). The capacity of each Affigel-10® folate-binding protein (FBP) column (Affigel-FBP columns) was determined by applying ten, 1 mL aliquots of 10 nmol/mL folic acid standard (Sigma® Chemical Co., St. Louis, MO) in 0.1 M potassium phosphate buffer (pH 7.0) at a flow rate of 0.3 mL/minute. Each 1 mL fraction was collected and saved. Five milliliters of 0.1 N HCl were added to each

column and the fractions were collected in 10 mL volumetric flasks. The fractions collected were brought to volume with additional 0.1 N HCl. The columns were flushed with an additional 5 mL of 0.1 N HCl, and these fractions were discarded. The columns were equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) and stored in the refrigerator with the column bed filled with the same buffer solution.

The absorbance of each 1 mL fraction was measured spectrophotometrically at a wavelength of 282 nm. The 10 mL fractions were measured at 296 nm. This information was used to determine the amount of folate that would saturate the columns and the amount of folate that would bind to the columns, respectively.

#### Purification and treatment of urine samples for HPLC.

Urine samples were defrosted, mixed well and adjusted to pH 7.0 with 5 N NaOH. The urine was filtered using Buchner funnels, #1 Whatman filter paper and a vacuum pump. Forty to 85 mL of urine were applied to an Affigel-FBP column that had been previously flushed with 5 mL of 0.1 N HCl and equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). (The amount of urine used varied depending on the expected urinary folate concentration.) A peristaltic pump was used to maintain a constant flow rate of 0.3 mL/min. After all of the sample had been applied to the column, the column was rinsed with 5 mL of 0.25 M potassium phosphate buffer containing

1.0 M NaCl (pH 7.0), followed by 5 mL of the buffer without NaCl. The folate bound to the column was eluted with 0.1 N HCl, with the first milliliter discarded. The eluted folate was brought up to a volume of 5 mL with 0.1 N HCl. The Affigel-FBP column was flushed with 5 mL of 0.1 N HCl, equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) and stored in the refrigerator with the column bed filled with the same buffer.

Chemical cleavage of folate to pABG. A 2 mL portion of the folate-containing solution eluted from the Affigel-FBP column was gently agitated and exposed to air for 30 minutes. Under these conditions, tetrahydrofolate is spontaneously cleaved by oxidation. Potassium acetate (2 N) was added to the sample, followed by the addition of 1 N NaOH, to bring the sample to a pH of 6.0. Five percent (v/v) hydrogen peroxide at 0.05 volume was added to oxidize N<sup>5</sup>-methyl-tetrahydrofolate to N<sup>5</sup>-methyl-dihydrofolate. Thirty seconds later, 0.1% bovine hepatic catalase (Sigma Chemical Co., St. Louis, MO) was added at 0.1 volume to decompose the remaining hydrogen peroxide. The N<sup>5</sup>-methyl-dihydrofolate was cleaved by acidifying the sample with 5 N HCl (0.1 volume). Cleavage of the folic acid present in the sample was achieved by the addition of a 0.1 mL zinc dust suspended in gelatin (1 g zinc dust in 4 mL 0.5% aqueous gelatin). The cleaved product was centrifuged to sediment the zinc. Finally, the sample was placed in an Amicon® MPS filtration unit with a YMT membrane (Amicon

Division, W.R. Grace & Co., Danvers, MA) and centrifuged at 1000g for 20 minutes to remove the gelatin and catalase.

HPLC Detection of pABG. High-performance liquid chromatography separation was performed using a Beckman Ultrasphere column and 0.1 M formic acid/2.5% acetonitrile mobile phase. Para-aminobenzoyl-L-glutamic acid (Sigma® Chemical Co., St Louis, MO), dissolved in 0.1 N NaOH to a final concentration of 5  $\mu$ M, was used as the standard. Detection was achieved with an absorbance monitor set at 280 nm. Two milliliters of each filtered, cleaved sample were loaded onto the HPLC column. Elution of the pABG fractions occurred in about 10 to 15 minutes, at which time the fractions were collected in 5 mL reaction vials. The pABG fractions were flushed with nitrogen gas and stored at -20° C.

Derivatization of pABG for GCMS. The pABG fractions were dried under nitrogen gas in a Reacti-Therm heating block and incubated with trifluoroacetic anhydride and trifluoroethanol at 90° C for one hour. The reagents were then evaporated under nitrogen gas and the derivatized pABG samples were stored in reaction vials at -20° C.

GCMS detection of derivatized pABG. The derivatized pABG samples were defrosted and dissolved in 100  $\mu$ L ethyl acetate. The samples were injected into the instrument and separated by gas chromatography. The carrier gas was helium. Derivatives of unlabeled and labeled pABG were analyzed by mass spectral monitoring (mass to charge ratios of 426 and 428,

respectively) using the negative-ion electron-capture chemical ionization mode with methane at 0.4 Torr as the reagent gas. The ratios of unlabeled/labeled pABG, as measured by GCMS, and the total urinary folate concentrations determined by the microbiological assay, were used to calculate the total urinary excretion of unlabeled and labeled folates.

### Statistical Analysis

Since the primary objective of this study was to determine the effect of supplemental folic acid on zinc status, computations for statistical power were based on univariate information of interindividual variation for plasma zinc and erythrocyte metallothionein concentrations. Using Student's two-sided t-test and assuming independence between successive measurements on the same individual, it was determined that a sample size of six subjects per group would provide 80% power to detect a meaningful change in plasma zinc concentrations. This same sample size provided 75% power in the case of erythrocyte metallothionein concentrations. These power calculations represented a conservative estimate since independence was assumed in a setting in which it was likely that positive correlations between successive measurements on a given subject would be observed.

Student's T-test was used to test for differences in age; weight; and usual intake of calories, protein, folate and zinc between subjects fed the zinc-adequate or zinc-restricted



diet. Analysis of variance for repeated measures was used to test for significant differences in mean values and slopes within and between the diet groups due to the effect of folic acid supplementation and level of zinc intake over time. The Statistical Analysis System (SAS Institute, Inc., 1989, Cary, NC) computer package was used for all analyses.

## CHAPTER 5 RESULTS

The results of this study are reported in three sections. The first section describes results that are relevant to both objectives of the study. This is followed by a description of the findings pertaining to the first and second objectives of the study, respectively.

No significant difference ( $P \geq 0.05$ ) was detected in the mean ( $\pm$  SD) age of subjects in the zinc-restricted ( $28.3 \pm 3.8$  y) versus the zinc-adequate diet groups ( $25.0 \pm 4.0$  y), nor were significant differences ( $P \geq 0.05$ ) detected in the average baseline weights of subjects (Table 5-1) within or between the study periods. Comparisons of the change in average weights, within or between study periods one and two, failed to detect significant differences ( $P \geq 0.05$ ) as well. The results of the complete blood counts with differentials and the 25-item blood chemistry profiles obtained at the beginning of both phases of the study were within normal limits for each subject.

Analysis of data obtained from food records (Table 5-2) did not reveal a significant difference ( $P \geq 0.05$ ) in the usual mean intake of calories or folate between the zinc diet groups; however, there were significant differences in the

usual mean intakes of protein ( $P < 0.05$ ) and zinc ( $P < 0.05$ ). The difference in the mean protein intake (i.e. 18 g) was equivalent to the protein content of a 2.5 oz portion of meat. If this was a 2.5 oz serving of red meat, it would essentially account for the difference in mean usual zinc intake (i.e. 2.8 mg) between the zinc diet groups. These differences

Table 5-1. Mean weights ( $\pm$  SD) of subjects on zinc-restricted or zinc-adequate diets.

<u>Study period I</u>		
	<u>Day 1</u>	<u>Day 29</u>
Zn-restricted	68.7 $\pm$ 6.4	69.5 $\pm$ 6.2
Zn-adequate	74.0 $\pm$ 13.7	73.7 $\pm$ 12.4
<u>Study period II</u>		
Zn-restricted	68.9 $\pm$ 5.9	69.4 $\pm$ 5.2
Zn-adequate	74.4 $\pm$ 13.0	74.2 $\pm$ 11.4

Table 5-2. Mean ( $\pm$  SD) usual calorie, protein, folate and zinc (Zn) intakes of subjects on zinc-restricted or zinc-adequate diets.

	<u>Zn-restricted</u>	<u>Zn-adequate</u>
Calories (kcal/d)	2313 $\pm$ 215	2556 $\pm$ 257
Protein (g/d)	98.5 $\pm$ 15.2	116.8 $\pm$ 11.7
Folate ( $\mu$ g/d)	306 $\pm$ 202	243 $\pm$ 49
Zinc (mg/d)	11.4 $\pm$ 0.9	14.2 $\pm$ 2.2

were not considered to be clinically significant since the mean protein intake of the zinc-restricted (i.e. 1.4 g/kg body weight) and zinc-adequate (i.e. 1.6 g/kg body weight) groups exceeded the RDA, and the mean usual zinc intakes exceeded 75% of the RDA for both groups. Additionally, the group with the lower mean usual zinc intake was the same group that received the zinc-restricted diet.

All subjects completed both phases of the study, and the results of the daily checklists, as well as the anonymous questionnaire completed at the end of the study revealed satisfactory compliance to the feeding regimen and study protocol. None of the subjects correctly identified the combination and order of treatments they received suggesting that they were adequately blinded to the treatments.

The mean ( $\pm$  SD) zinc content of the diet fed to the subjects during study periods one and two, as determined by direct analysis, was  $3.5 \pm 0.2$  mg zinc/d. The accuracy and precision of the method used to determine the zinc content of the diet were verified by concurrent analysis of citrus leaves certified for zinc content by the National Institute of Standards and Technology. The published reference value for this material is  $29 \pm 2$   $\mu$ g/g, and the mean laboratory value obtained was  $30 \pm 1$   $\mu$ g/g. The interassay coefficient of variation was 3.3%.

The molar ratio of zinc to folic acid provided by the various treatment combinations is listed in Table 5-3. In all

cases, the molar ratios were lower than the ratio obtained using the 1989 RDA (Food and Nutrition Board, 1989a; 1989b) for zinc and folate (i.e. 511:1).

Table 5-3. Molar ratios of zinc (Zn) to folic acid (FA) for each treatment combination.

<u>Treatment combination</u>	<u>Molar ratio (Zn:FA)</u>
Zn-restricted, no supplemental FA	82:1
Zn-restricted, 800 $\mu$ g supplemental FA	30:1
Zn-adequate, no supplemental FA	338:1
Zn-adequate, 800 $\mu$ g supplemental FA	123:1

#### Effect of Supplemental Folic Acid on Zinc Status

The response variables measured to determine if supplemental folic acid affects zinc status in human subjects consuming zinc-restricted or zinc-adequate diets included: plasma zinc, erythrocyte zinc, erythrocyte metallothionein, serum alkaline phosphatase and urinary zinc. Serum ferritin concentrations were also measured in order to determine if the erythrocyte metallothionein response was specific or due to changes in iron metabolism. Analysis of variance for repeated measures was used to test for differences in the means for each response variable, within and between the zinc diet groups, due to the effect of the supplement (i.e. the response on the supplement minus the response off the supplement). The same statistical procedure was used to test for differences in

the mean response over time due to the effect of the supplement.

### Plasma Zinc

The accuracy and precision of the method used to determine plasma zinc concentrations were evaluated by concurrent analysis of National Institute of Standards and Technology bovine serum and pooled plasma samples. The mean ( $\pm$  SD) value for the bovine serum was  $93 \pm 0 \mu\text{g/dL}$  compared with the published value of  $92 \pm 6 \mu\text{g/dL}$ . The interassay coefficient of variation was 4.0%, and the average intraassay coefficient of variation was 3.4%.

No significant differences ( $P \geq 0.05$ ) were detected in mean plasma zinc values (Table 5-4) within or between the zinc diet groups due to the effect of the supplement, and plasma zinc values were within the normal range (i.e.  $\geq 70 \mu\text{g/dL}$ ) (Gibson, 1990) for all but one subject. The subnormal plasma zinc values reported for subject #5 occurred during phase two of the study when he was consuming the zinc-restricted diet without supplemental folic acid. The initial baseline value for this subject during study period two was  $62 \mu\text{g/dL}$ . This value declined to a minimum of  $41 \mu\text{g/dL}$  about midway through the study period and then increased to a final value of  $58 \mu\text{g/dL}$ . The mean plasma zinc values for this subject during study periods one and two ( $89 \pm 4$  versus  $53 \pm 9 \mu\text{g/dL}$ , respectively) were significantly different ( $P < 0.05$ ). This

subject also displayed behavior suggestive of depression and/or irritability.

Table 5-4. Overall mean ( $\pm$  SD) values of response variables used to assess the effect of supplemental folic acid (FA) on zinc status in subjects consuming zinc-restricted or zinc-adequate diets.

<u>Response variables</u>	<u>Zn intake</u>	<u>FA treatment</u>	
		<u>+FA</u>	<u>-FA</u>
Plasma zinc ( $\mu\text{g/dL}$ )	Restricted	82 $\pm$ 5	81 $\pm$ 14
	Adequate	86 $\pm$ 6	86 $\pm$ 10
Erythrocyte zinc ( $\mu\text{g/g}$ protein)	Restricted	37 $\pm$ 3	40 $\pm$ 3
	Adequate	38 $\pm$ 5	38 $\pm$ 6
Serum alkaline phosphatase (U/L)	Restricted	87 $\pm$ 11	92 $\pm$ 15
	Adequate	93 $\pm$ 21	100 $\pm$ 27
Erythrocyte MT ( $\mu\text{g/g}$ protein)	Restricted	14 $\pm$ 2	12 $\pm$ 2
	Adequate	13 $\pm$ 3	12 $\pm$ 2
Serum ferritin (ng/mL)	Restricted	101 $\pm$ 45	109 $\pm$ 58
	Adequate	128 $\pm$ 40	114 $\pm$ 72
Urinary zinc ( $\mu\text{g/24 h}$ )	Restricted	384 $\pm$ 138	539 $\pm$ 281
	Adequate	652 $\pm$ 406	597 $\pm$ 446

The effect of supplemental folic acid on plasma zinc in subjects consuming the zinc-restricted or zinc-adequate diets over time is illustrated in Figure 5-1. No significant ( $P \geq 0.05$ ) time  $\times$  supplement  $\times$  diet interaction was detected. Comparison of the slopes of the lines for the zinc-restricted and zinc-adequate groups, due to the effect of the supplement (i.e. on minus off the supplement), revealed the lack of a

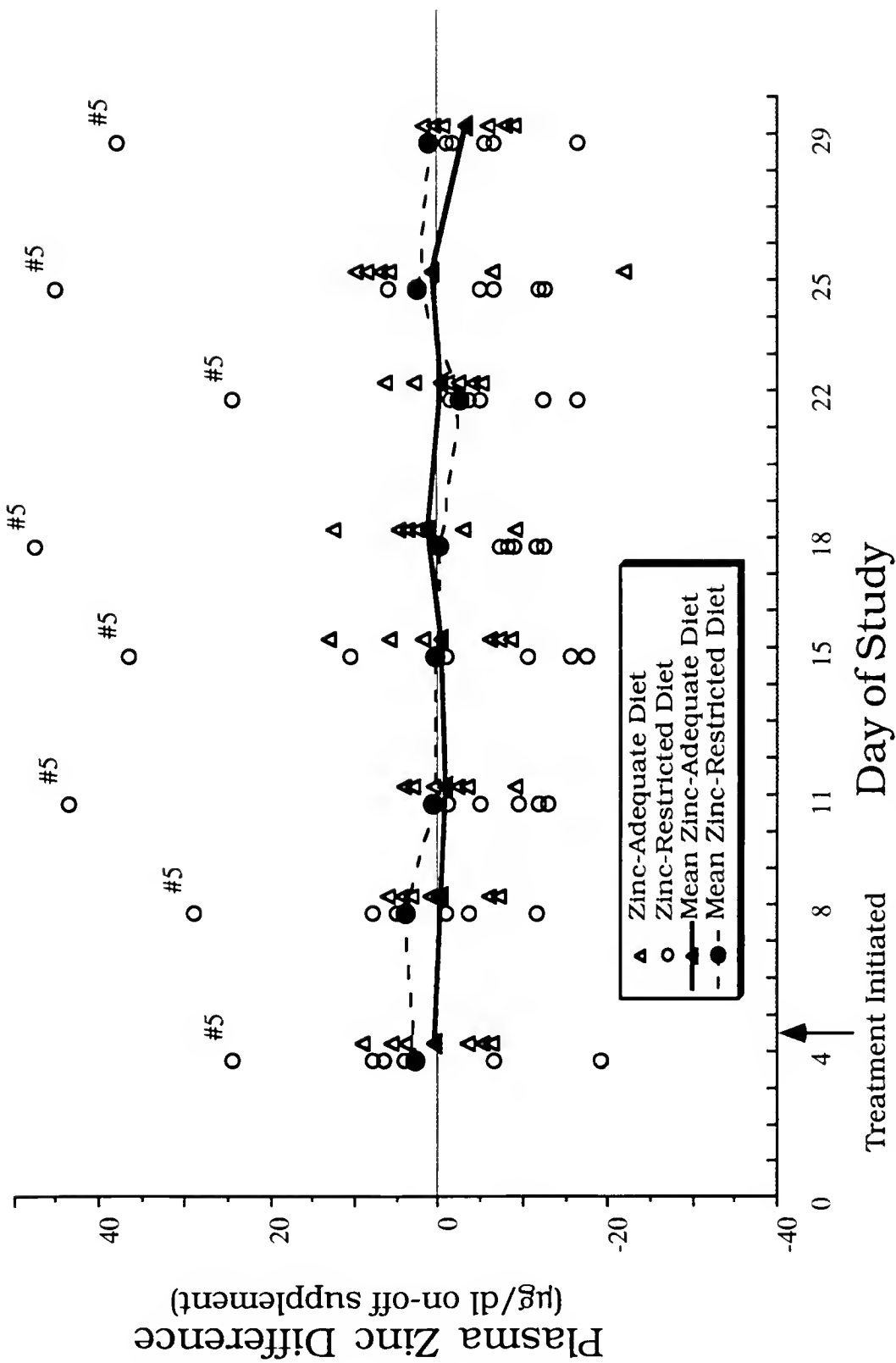


Figure 5-1. Effect of supplemental folic acid on plasma zinc in subjects consuming zinc-restricted or zinc-adequate diets.



statistically significant difference ( $P \geq 0.05$ ) as well. This finding suggests the lack of a supplemental effect due to the level of zinc intake.

Within the zinc-restricted group, no significant difference ( $P \geq 0.05$ ) was detected in the slopes of the lines when subjects received the supplement versus the placebo. Mean plasma zinc concentrations on and off the supplement were similar, as can be seen by the fact that the line representing the mean difference values for this group was almost superimposed on the zero axis (Figure 5-1). The same was true for the zinc-adequate group.

#### Erythrocyte Zinc

No significant differences ( $P \geq 0.05$ ) in mean erythrocyte zinc values (Table 5-4) were detected within or between the zinc diet groups due to the effect of the supplement. Unlike plasma zinc, significant differences ( $P \geq 0.05$ ) in mean erythrocyte zinc concentrations were not detected for subject #5 during study periods one and two (i.e.  $39 \pm 4$  versus  $36 \pm 3$   $\mu\text{g/g}$  protein).

As illustrated by the lack of contrast in the patterns of change over time (Figure 5-2), a significant time x supplement x diet interaction was not detected ( $P \geq 0.05$ ) for this response variable. Also, no significant differences ( $P \geq 0.05$ ) in the change in erythrocyte zinc concentrations were detected

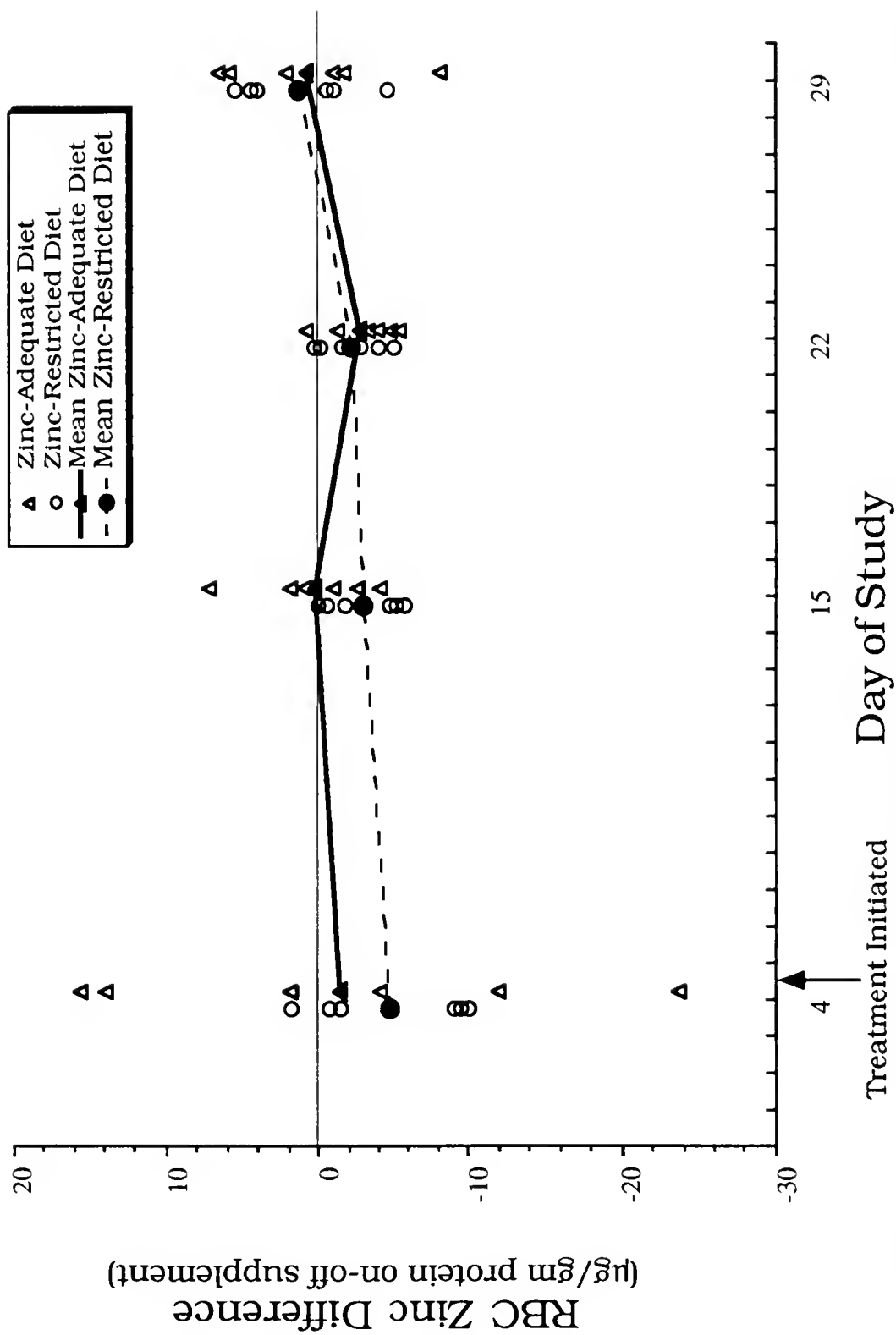


Figure 5-2. Effect of supplemental folic acid on erythrocyte zinc in subjects consuming zinc-restricted or zinc-adequate diets.

between or within the zinc diet groups due to the effect of the supplement.

The precision of the method used to determine erythrocyte zinc concentrations was assessed by analyzing a pooled erythrocyte lysate sample. The interassay coefficient of variation of 3.4%, and the average intraassay coefficient of variation was 1.8%.

#### Serum Alkaline Phosphatase

No significant differences ( $P \geq 0.05$ ) were detected in mean serum alkaline phosphatase values (Table 5-4) within or between the zinc diet groups, and with the exception of subject #11, serum alkaline phosphatase values were within normal limits (20 to 140 U/L, SmithKline Beecham Clinical Laboratories, Gainesville, FL) during both study periods. During study period two, subject #11 had a baseline value of 151 U/L, which declined to 136 and 139 U/L on days 15 and 29, respectively. The latter two values were comparable to his prestudy value of 138 U/L. During study period one, subject #11's baseline value was 122 U/L. This value declined to 118 and 98 U/L on days 15 and 29, respectively. The larger decline in values observed from days 15 to 29 during study period one may have been related to the low grade fever (99.7 °F to 101.3 °F) experienced by this subject on days 23 through 27, for which he was treated with acetaminophen.

The effect of supplemental folic acid on serum alkaline phosphatase over time is shown in Figure 5-3. Although it was observed that some individuals in both diet groups had larger baseline difference values, their difference values at subsequent time intervals tended to be smaller and were clustered near the zero axis. Exceptions to this phenomenon were subjects #4 and #11. Subject #4 had large difference values throughout the study; however, his pattern of change was similar to the overall pattern for the zinc-restricted group. As discussed above, subject #11 responded much more negatively to the effect of the supplement between days 15 and 29 compared to the rest of the subjects in the zinc-adequate group. Consequently, the line connecting the mean serum alkaline phosphatase difference values for subjects consuming the zinc-adequate diet had a downward trend at the conclusion of the study. This line would have been parallel to the line representing the means for the zinc-restricted group if subject #11's data for day 29 had been omitted. Despite these somewhat different patterns of change, no significant ( $P \geq 0.05$ ) time x supplement x diet interaction was detected. No significant difference ( $P \geq 0.05$ ) in the change in serum alkaline phosphatase values due to the effect of the supplement were detected between the zinc diet groups; nor were significant differences detected ( $P \geq 0.05$ ) when the change in this response variable on versus off the supplement was compared within each of the diet groups.

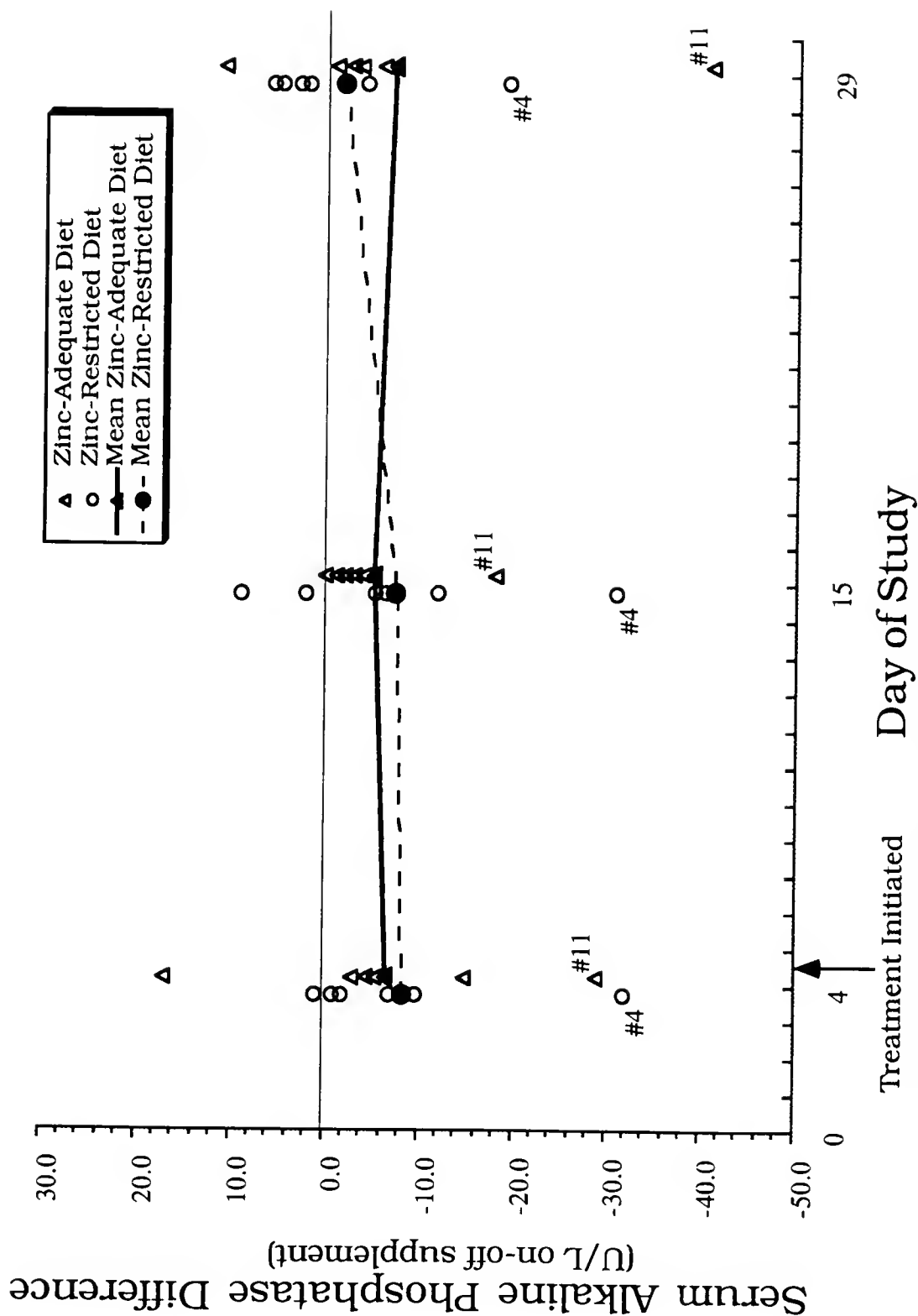


Figure 5-3. Effect of supplemental folic acid on serum alkaline phosphatase in subjects consuming zinc-restricted or zinc-adequate diets.

### Erythrocyte Metallothionein

Baseline, and all successive values for erythrocyte metallothionein were below the mean value ( $\pm$  SD) previously reported for a group of 44 subjects (i.e.  $47.5 \pm 30.0 \mu\text{g/g}$  protein; Thomas et al., 1992). The reason for these differences is unknown, although it is possible that the variation in results occurred due to the use of standards prepared at different points in time.

The precision of the method, using a pooled erythrocyte lysate sample, could not be adequately evaluated because the values obtained for this sample could not be read within the limits of the standard curve. The absorbance readings suggested that the erythrocyte metallothionein concentrations of the pooled sample were very low. It is possible that the concentration of metallothionein in this sample declined during storage due to protein degradation (Davies and Goldberg, 1987). Unlike the pooled sample, it was possible to read the subjects' samples within the limits of the standard curve.

In an attempt to ascertain the approximate degree of interassay variability, the interassay coefficient of variation was calculated using the adjusted absorbance readings of the pooled sample (i.e. absorbance reading minus the blank). This value was 18.6%. The coefficients of variation, based on the slope and intercept of the standard

curve for each run of the assay, were 14.0% and 8.3%, respectively.

There was a significant ( $P < 0.001$ ) time x supplement x diet interaction for this response variable as illustrated in Figure 5-4. For subjects consuming the zinc-restricted diet, the supplemental effect resulted in a gradual increase in erythrocyte metallothionein difference values, followed by a decline. The effect of folic acid supplementation in the zinc-adequate group resulted in a slight decrease in erythrocyte metallothionein difference values over time. The continued negative direction of the line connecting the mean erythrocyte metallothionein difference values for the zinc-adequate group (i.e days 22 through 29) was due to the large difference value observed on day 29 for subject #8. In comparison, by day 29, the rest of the subjects in the zinc-adequate group had difference values clustered around the zero axis. No significant differences ( $P \geq 0.05$ ) were detected when the slopes of the lines on the supplement versus the slopes of the lines on the placebo were compared within the zinc-restricted and zinc-adequate diet groups. Comparisons of the mean erythrocyte metallothionein concentrations (Table 5-4) within and between the zinc diet groups failed to produce significant differences ( $P \geq 0.05$ ) as well.

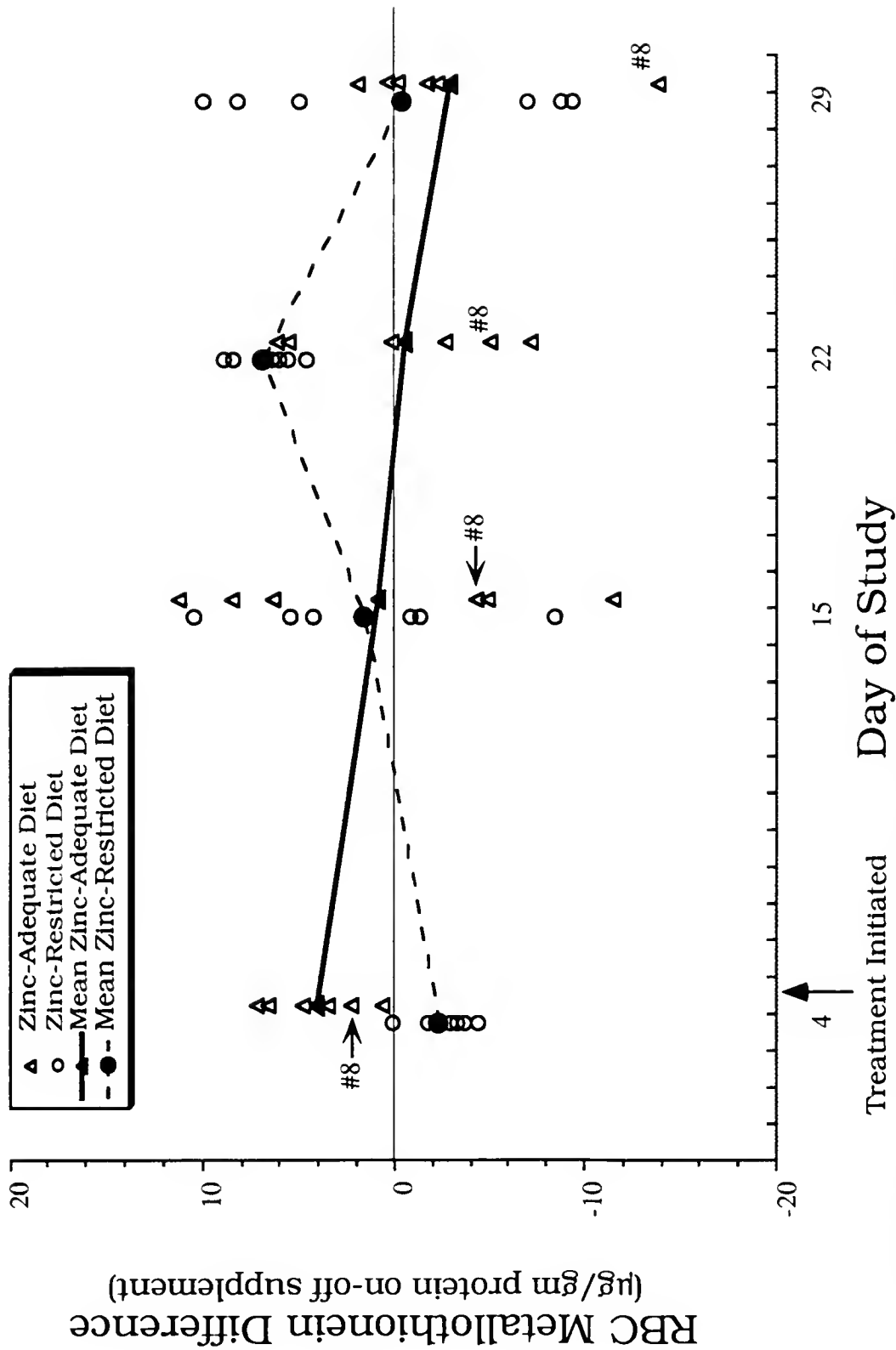


Figure 5-4. Effect of supplemental folic acid on erythrocyte metallothionein in subjects consuming zinc-restricted or zinc-adequate diets.



### Serum Ferritin

Individual serum ferritin concentrations tended to decline over the course of each study period, and compared with study period one, baseline values were observed to be lower during study period two. These trends may have been due to the frequency of phlebotomy and the volume of blood obtained. Despite these trends, serum ferritin concentrations were within normal limits (i.e. 18 to 300 ng/mL) (Young, 1987) for all but one subject during both study periods. The exception was subject #5 whose serum ferritin concentrations during study period two (19, 15 and 27 ng/mL) were suggestive of borderline deficiency. Corresponding values during study period one were 175, 136 and 106 ng/mL. When mean serum ferritin values (Table 5-4) within and between the zinc diet groups were compared, no significant differences ( $P \geq 0.05$ ) were detected.

Figure 5-5 shows the effect of supplemental folic acid on serum ferritin. Compared to the zinc-restricted group, a slight upward trend in the line connecting the mean difference values for the zinc-adequate group was observed between days 15 and 29; however, no significant ( $P \geq 0.05$ ) time x supplement x diet interaction was detected.

Despite the slight upward trend noted for the zinc-adequate group, no significant difference ( $P \geq 0.05$ ) was detected due to the effect of the supplement between the zinc diet groups. This trend was due to the large difference

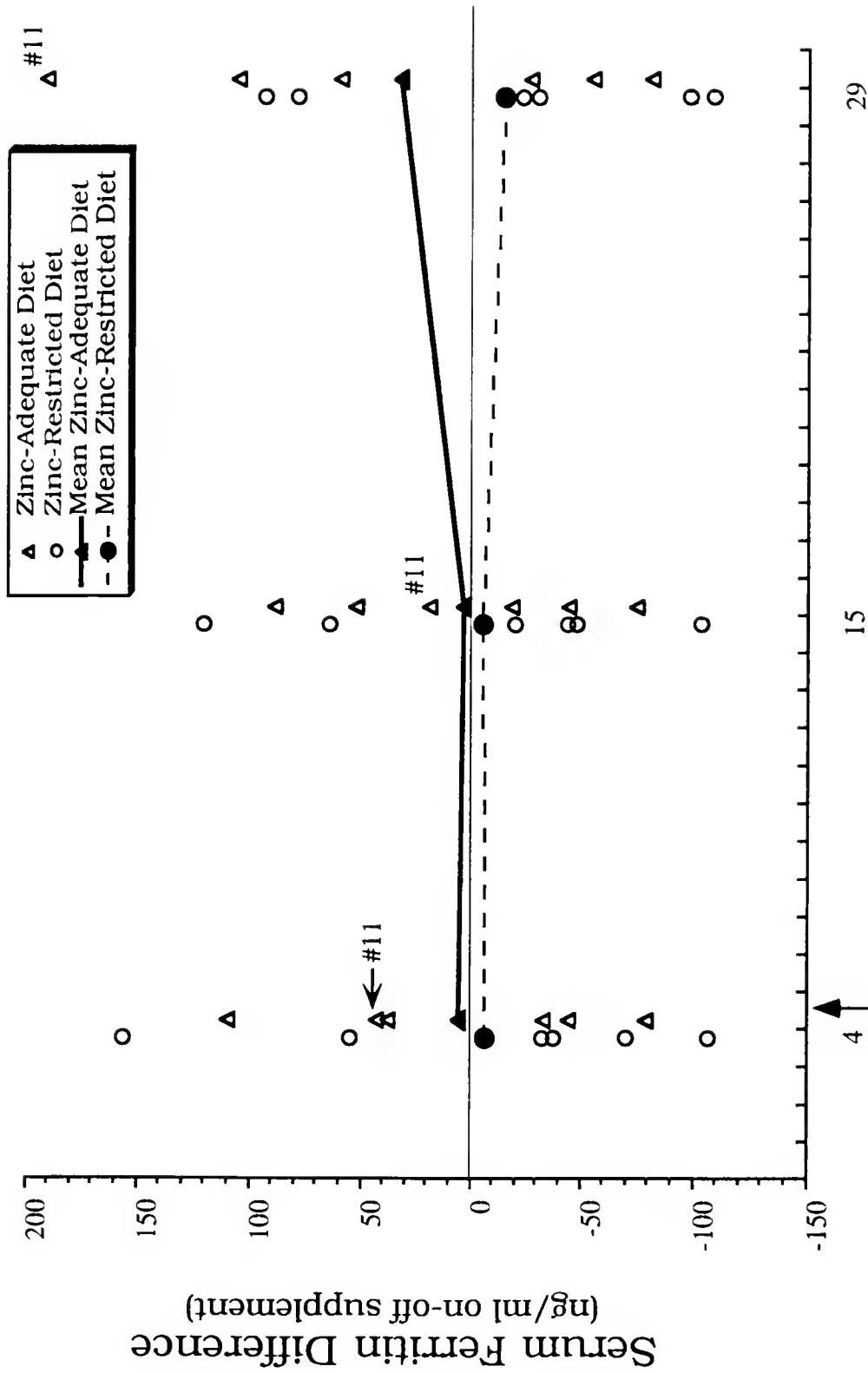


Figure 5-5. Effect of supplemental folic acid on serum ferritin in subjects consuming zinc-restricted or zinc-adequate diets.

value obtained for subject #11 on day 29. This subject's serum ferritin values were 80, 50 and 209 ng/mL when he received the supplement and 37, 31 and 18 ng/mL when he received the placebo (i.e. study periods one and two, respectively). The large increase from day 15 to day 29 during study period one, which was not replicated during study period two, may have been related to the low grade fever experienced by this subject rather than to the effect of the supplement, since serum ferritin concentrations are known to increase with infection (Reeves and Haurani, 1980). When the effect of the supplement versus the placebo was compared within each level of zinc intake, there were no significant differences ( $P \geq 0.05$ ) in the slopes of the lines for either of the groups.

#### Urinary Zinc

The accuracy and precision of the method used to determine urinary zinc concentrations were assessed by simultaneous analysis of Gilford Urine Metal Control (Ciba Corning Diagnostics Corporation, Irvine, CA). The mean value obtained for this control was  $100 \pm 0.6 \mu\text{g/dL}$ , compared to the published target concentration of  $100 \mu\text{g/dL}$ . The interassay coefficient of variation was 0.6%, and the average intraassay coefficient of variation was 0.5%.

No significant differences ( $P \geq 0.05$ ) in mean urinary zinc values (Table 5-4) were detected within or between the zinc diet groups due to the effect of the supplement. Baseline

urinary zinc values ranged from 164 to 1235  $\mu\text{g}/24\text{ h}$ , which is similar to the normal range (150 to 1200  $\mu\text{g}/24\text{ h}$ ) (Young, 1987). Urinary zinc concentrations dropped below the lower limit of normal for subject #5 during study period two. This coincided with the timing of the reduction observed in his plasma zinc concentrations. Below normal urinary zinc concentrations were also noted for two subjects consuming the zinc-adequate diet. Subject #10 had consistently low, yet relatively stable concentrations of zinc in his urine, regardless of whether he received the folic acid supplement or the placebo. Borderline low urinary zinc concentrations were observed in subject #7 only when he received the placebo. This was probably related to the fact that his baseline value was also much lower during the placebo period.

The effect of the supplement on urinary zinc in subjects consuming zinc-restricted or zinc-adequate diets over time is shown in Figure 5-6. No significant ( $P \geq 0.05$ ) time x supplement x diet interaction was detected. No significant difference ( $P \geq 0.05$ ) in the change in urinary zinc difference values was detected between the zinc diet groups either, suggesting the lack of an effect due to the supplement.

Subjects consuming the zinc-restricted diet responded to the reduction in zinc intake with significant decreases (placebo:  $P < 0.01$ ; supplement:  $P < 0.05$ ) in mean urinary zinc concentrations from baseline. However, no significant difference ( $P \geq 0.05$ ) was detected in the change in mean values

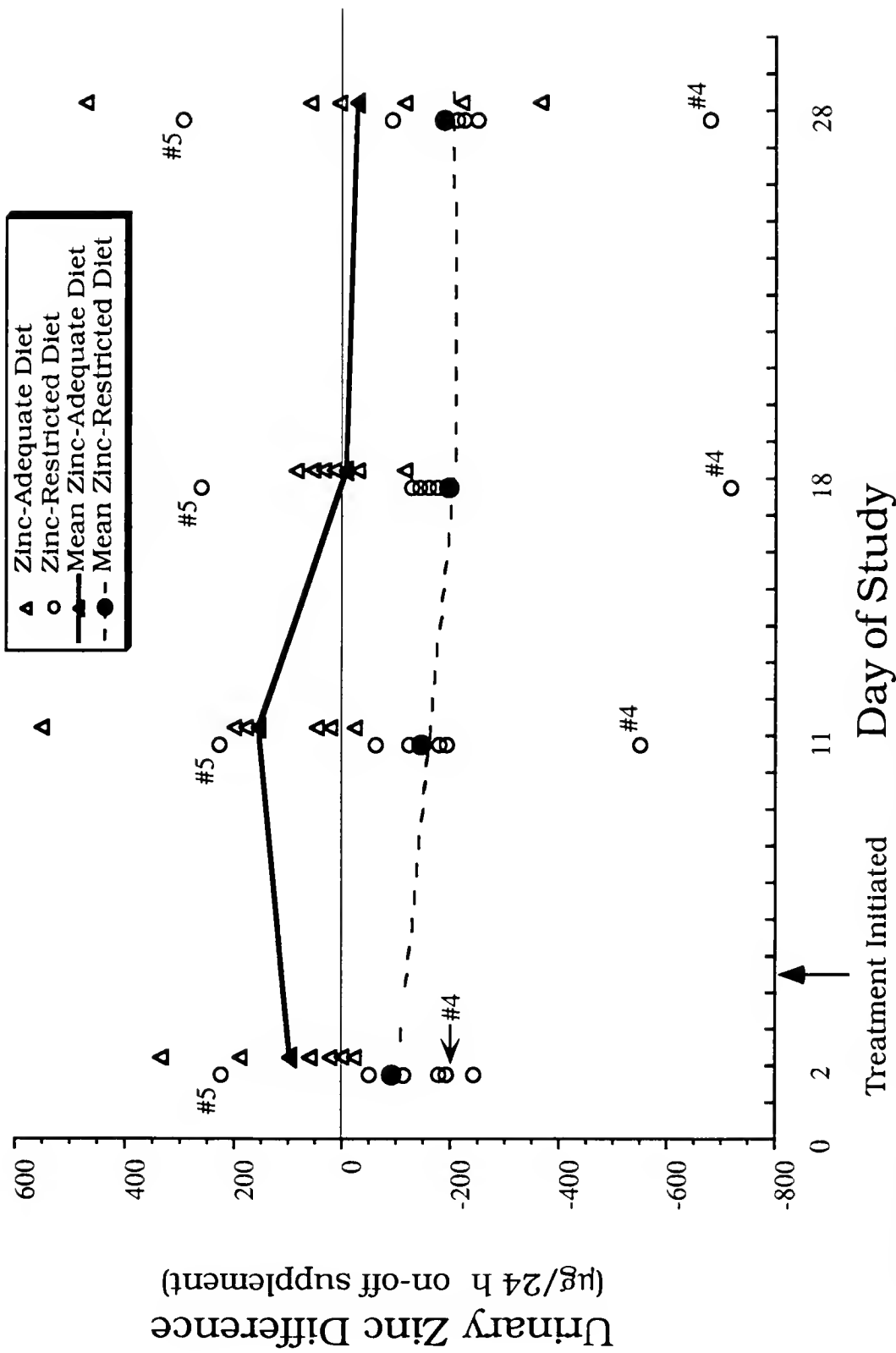


Figure 5-6. Effect of supplemental folic acid on urinary zinc in subjects consuming zinc-restricted or zinc-adequate diets.

on the supplement compared to the change on the placebo. Despite the lack of a statistically significant difference, two subjects (i.e. subjects #4 and #5) appeared to respond to the effect of the supplement differently than the others. The greater reduction in urinary zinc excretion that occurred when subject #4 received the supplement is illustrated in Figure 5-6 (days 11, 18 and 28). In contrast, subject #5 excreted less zinc in his urine when he received the placebo; however, compared to baseline, little difference in the change in his values on versus off the supplement was observed at any time point. This suggests that he responded similarly to the effects of a zinc-restricted diet during both phases of the study, but that his zinc status was more severely compromised at the beginning of study period two than study period one.

In contrast to the zinc-restricted group, no significant ( $P \geq 0.05$ ) change from baseline was detected for urinary zinc when subjects in the zinc-adequate group received the supplement or when they received the placebo. Furthermore, a significant difference ( $P \geq 0.05$ ) was not detected in the change in mean urinary zinc concentrations on the supplement compared to the change on the placebo.

#### Effect of Level of Zinc Intake on Folate Utilization

To determine the effect of zinc intake on folate utilization, total urinary, serum and erythrocyte folate concentrations, and urinary excretion of deuterium-labeled and

unlabeled folate were determined in subjects consuming zinc-restricted or zinc-adequate diets. Analysis of variance for repeated measures was used to test for differences in mean values and slopes when subjects in the zinc-restricted and zinc-adequate diet groups were receiving supplemental folic acid.

### Serum Folate

The precision of the method used to determine serum folate concentrations was evaluated by concurrent analysis of pooled serum samples with known concentrations. The interassay coefficients of variation were 5.8 and 8.2%, and the average intraassay coefficients of variation were 9.6 and 9.2% for the low and high pooled samples, respectively.

There was no evidence to suggest ( $P \geq 0.05$ ) that the mean serum folate response to folic acid supplementation (Table 5-5) was influenced by the level of zinc intake. As shown in Figure 5-7, both groups experienced a significant increase ( $P < 0.001$ ) in serum folate concentrations over the course of the study; however, no significant difference ( $P \geq 0.05$ ) was detected when the slope of the line for the zinc-restricted group was compared to the slope for the zinc-adequate group.

### Erythrocyte Folate

No significant difference ( $P \geq 0.05$ ) in the mean erythrocyte folate response to folic acid supplementation

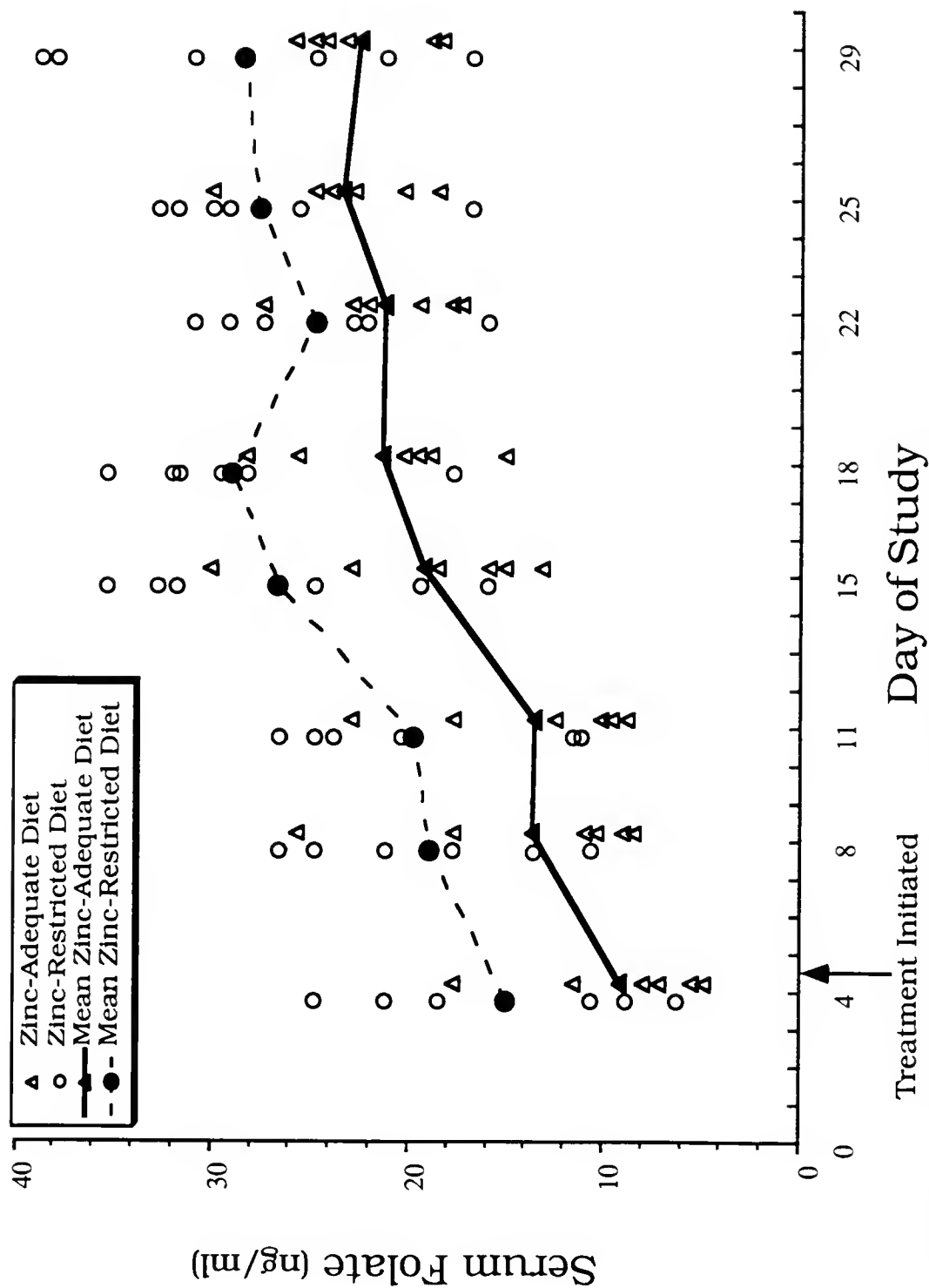




Table 5-5. Overall mean ( $\pm$ SD) values of response variables used to assess the response to folic acid (FA) supplementation in subjects consuming zinc-restricted or zinc-adequate diets.

<u>Response variables</u>	<u>+/-FA</u>	<u>Zn intake</u>	
		<u>3.5 mg</u>	<u>14.5 mg</u>
Serum folate (ng/mL)	+FA	23.8 $\pm$ 6.1	18.0 $\pm$ 4.5
	-FA	17.7 $\pm$ 2.6	15.3 $\pm$ 4.0
Erythrocyte folate (ng/mL)	+FA	375 $\pm$ 90	297 $\pm$ 98
	-FA	348 $\pm$ 77	300 $\pm$ 98
Urinary folate (total) ( $\mu$ g/24 h)	+FA	494 $\pm$ 300	424 $\pm$ 293
	-FA	128 $\pm$ 96	119 $\pm$ 84
Deuterium-labeled urinary folate ( $\mu$ g/24 h)	+FA	290 $\pm$ 80	259 $\pm$ 108
	-FA	not applicable	

(Table 5-5) was detected due to the level of zinc intake. The overall response to the supplement was positive (Figure 5-8), with both groups achieving statistically significant increases ( $P < 0.05$ , zinc-restricted;  $p < 0.01$ , zinc-adequate) in mean erythrocyte folate concentrations over the course of the study. However, no significant difference ( $P \geq 0.05$ ) was detected in the change in the mean erythrocyte folate response due to the level of zinc intake.

A pooled whole blood sample with a known folate concentration was used to assess the precision of the method. The interassay coefficient of variation was 8.1%, and the average intraassay coefficient of variation was 5.7%.

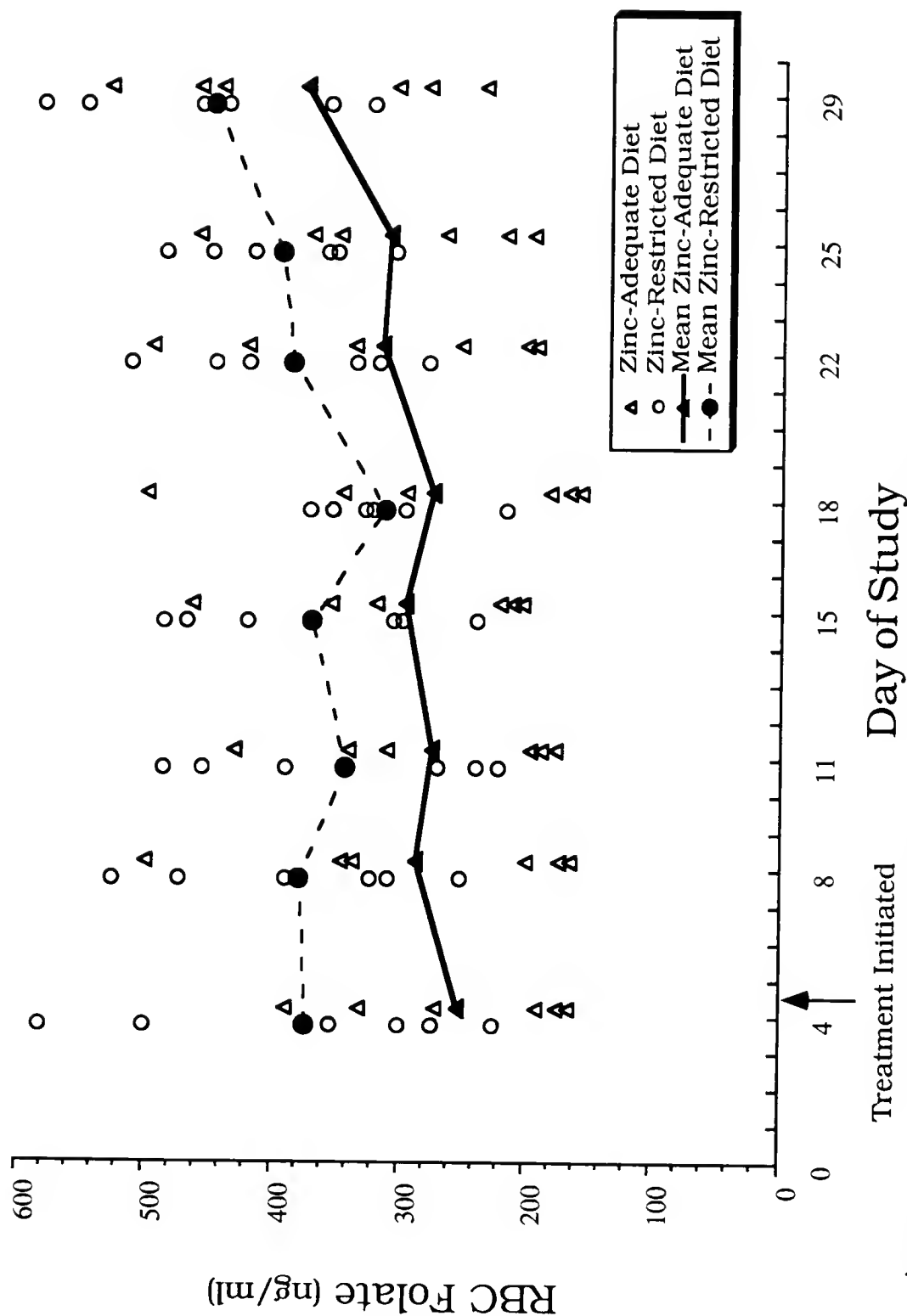


Figure 5-8. Erythrocyte folate response to folic acid supplementation in subjects consuming zinc-restricted or zinc-adequate diets.

### Urinary Folate

Total urinary folate. Pooled serum samples with known folate concentrations were used to assess the precision of the method used to determine urinary folate concentrations. The interassay coefficients of variation were 6.1 and 8.0%, and the average intraassay coefficients of variation were 9.4 and 12.2% for the low and high pooled samples, respectively.

There was no evidence ( $P \geq 0.05$ ) to suggest that the mean urinary folate response to folic acid supplementation (Table 5-5) was influenced by the level of zinc intake, and mean urinary folate concentrations for both groups of subjects were significantly higher ( $P < 0.001$ ) when they received the supplement versus the placebo. The large standard deviations obtained for both zinc diet groups during supplementation were due to the fact that folate excretion was much lower at baseline, after which it steadily increased in response to folic acid supplementation.

As depicted in Figure 5-9, there was no evidence ( $P \geq 0.05$ ) to support a difference in urinary folate response to folic acid supplementation due to the level of zinc intake. Within each of the zinc diet groups, the subjects experienced significantly greater increases ( $P < 0.001$ ) in urinary folate concentrations when they received the supplement versus the placebo.

Subject #9 tended to excrete less folate (both labeled and unlabeled) in his urine than other subjects, although the

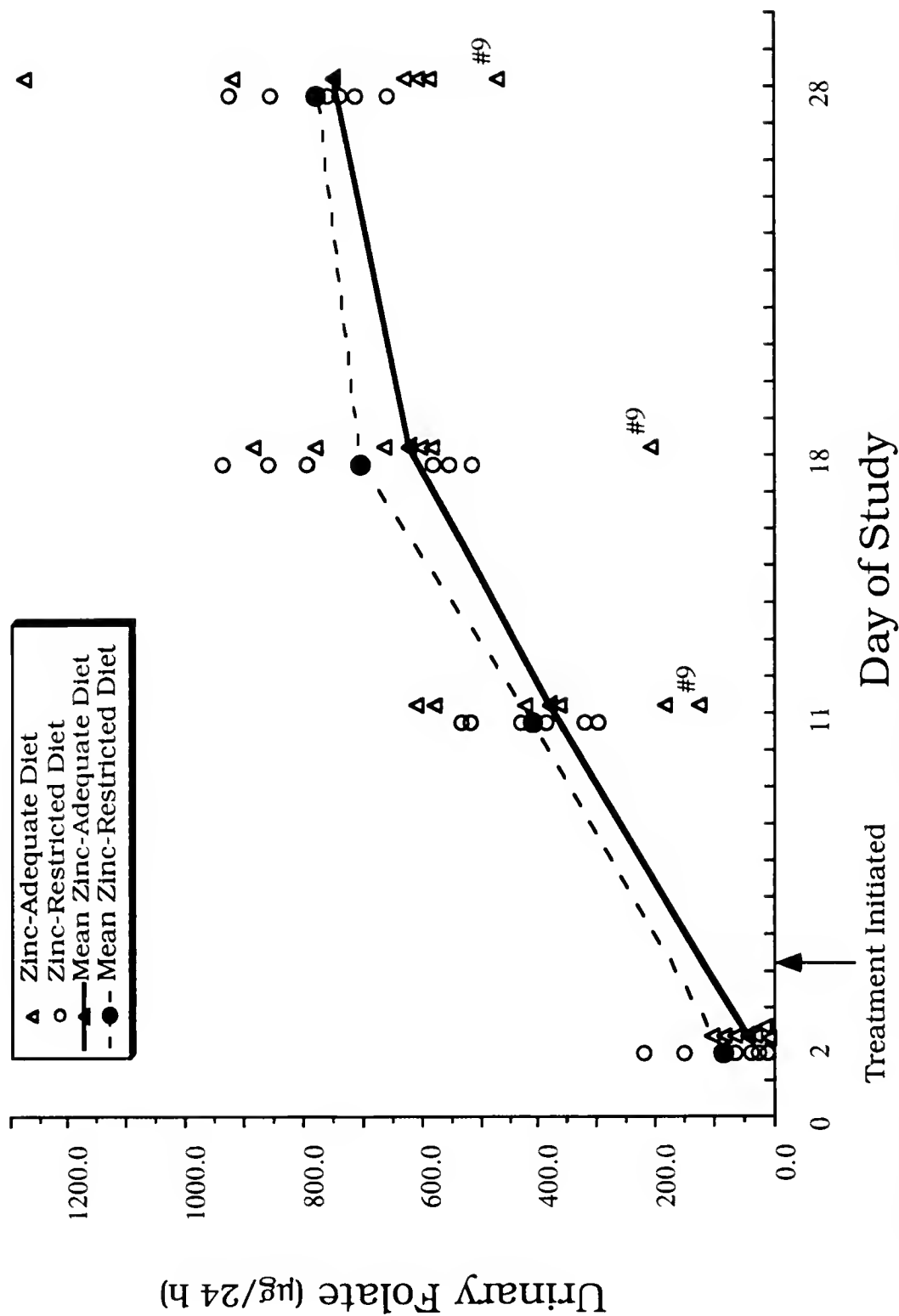


Figure 5-9. Urinary folate response to folic acid supplementation in subjects consuming zinc-restricted or zinc-adequate diets.

overall change in his excretion pattern was similar to that of the other subjects. This subject also tended to have low urinary folate concentrations when he received the placebo.

Urinary deuterium-labeled folate. Excretion of deuterium-labeled folate in response to folic acid supplementation is illustrated in Figure 5-10. Similar to the data for total urinary folate, there was no evidence to support a difference ( $P \geq 0.05$ ) in the urinary deuterium-labeled folate response to folic acid supplementation due to the level of zinc intake. There was also no significant difference ( $P \geq 0.05$ ) in the average amount of deuterium-labeled folate excreted (Table 5-5) due to the level of zinc intake. There was, however, a significant increase for both groups ( $P < 0.001$ ) in the amount of labeled folate excreted between days 11 and 18.

When the data were expressed as percentages of total folate intake (i.e. 290  $\mu\text{g/d}$  dietary folate, plus 800  $\mu\text{g/d}$  supplemental folic acid), no significant difference ( $P \geq 0.05$ ) was detected in the mean value (Table 5-6) obtained for the zinc-restricted versus the zinc-adequate diet groups. There was also no evidence to suggest ( $P \geq 0.05$ ) that the change in the percent of total folate intake excreted with the deuterium label was different due to the level of zinc intake (Figure 5-11). The same was true when the data were expressed as percentages of total urinary folate (Table 5-6; Figure 5-12) or as percentages of the oral dose (Table 5-6; Figure 5-13).

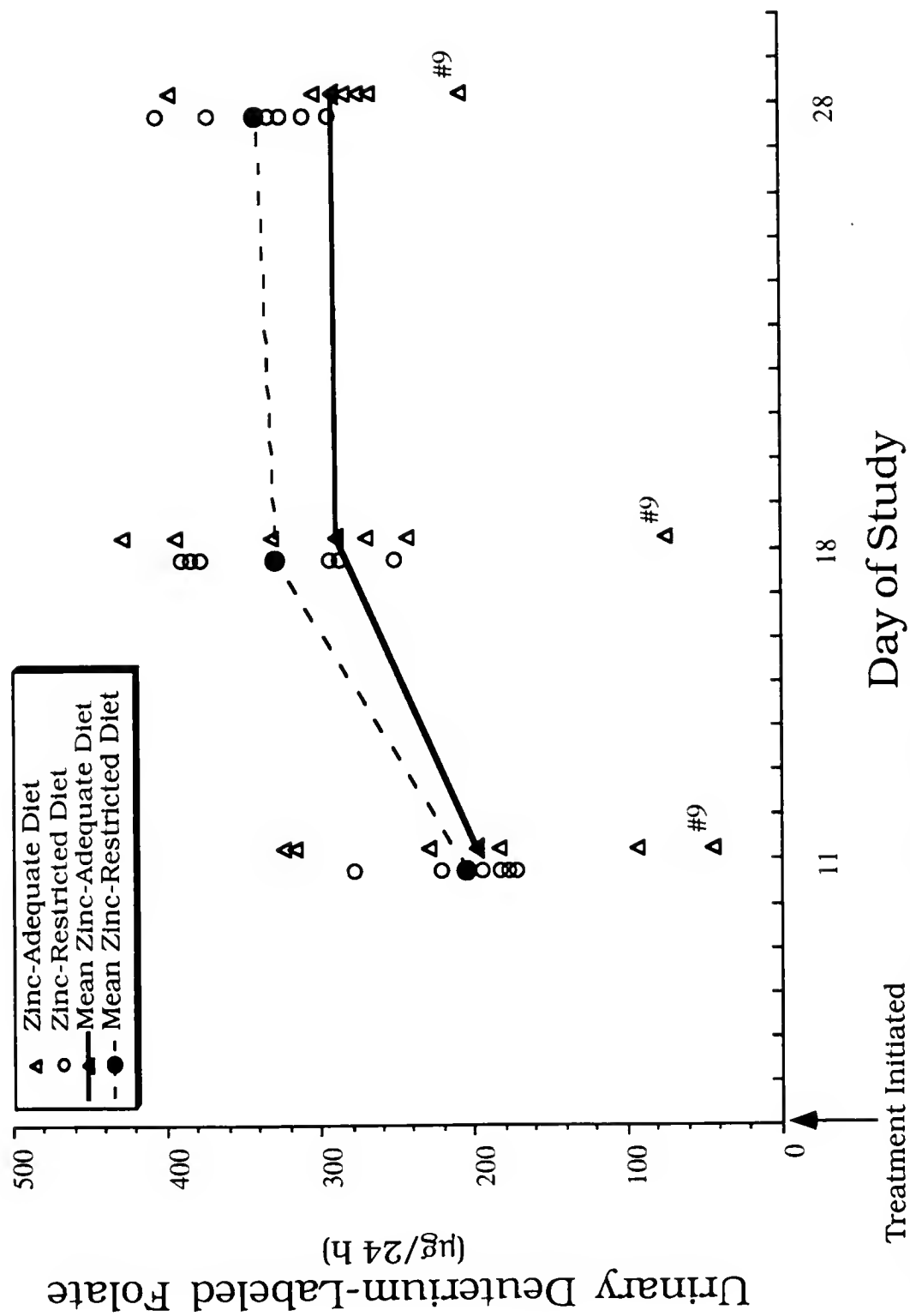


Figure 5-10. Urinary deuterium-labeled folate response to folic acid supplementation in subjects consuming zinc-restricted or zinc-adequate diets.

As shown in Figure 5-12, approximately 45% of the folate excreted in the urine was excreted as deuterium-labeled folate by day 28. Since 74% of the total folate intake was deuterium-labeled, equilibrium was not reached within the time frame of this study.

Table 5-6. Overall mean ( $\pm$ SD) deuterium-labeled folate ( $D_2FA$ ) excreted expressed as a percentage of total folate intake, total urinary folate and oral dose.

<u>Response variables</u>	<u>Zn intake</u>	
	<u>3.5 mg</u>	<u>14.5 mg</u>
$D_2FA$ /total folate intake (%)	26.7 $\pm$ 7.3	23.8 $\pm$ 9.8
$D_2FA$ /total urinary folate (%)	47.0 $\pm$ 5.1	46.6 $\pm$ 6.5
$D_2FA$ /oral dose (%)	36.3 $\pm$ 10.0	32.4 $\pm$ 13.3

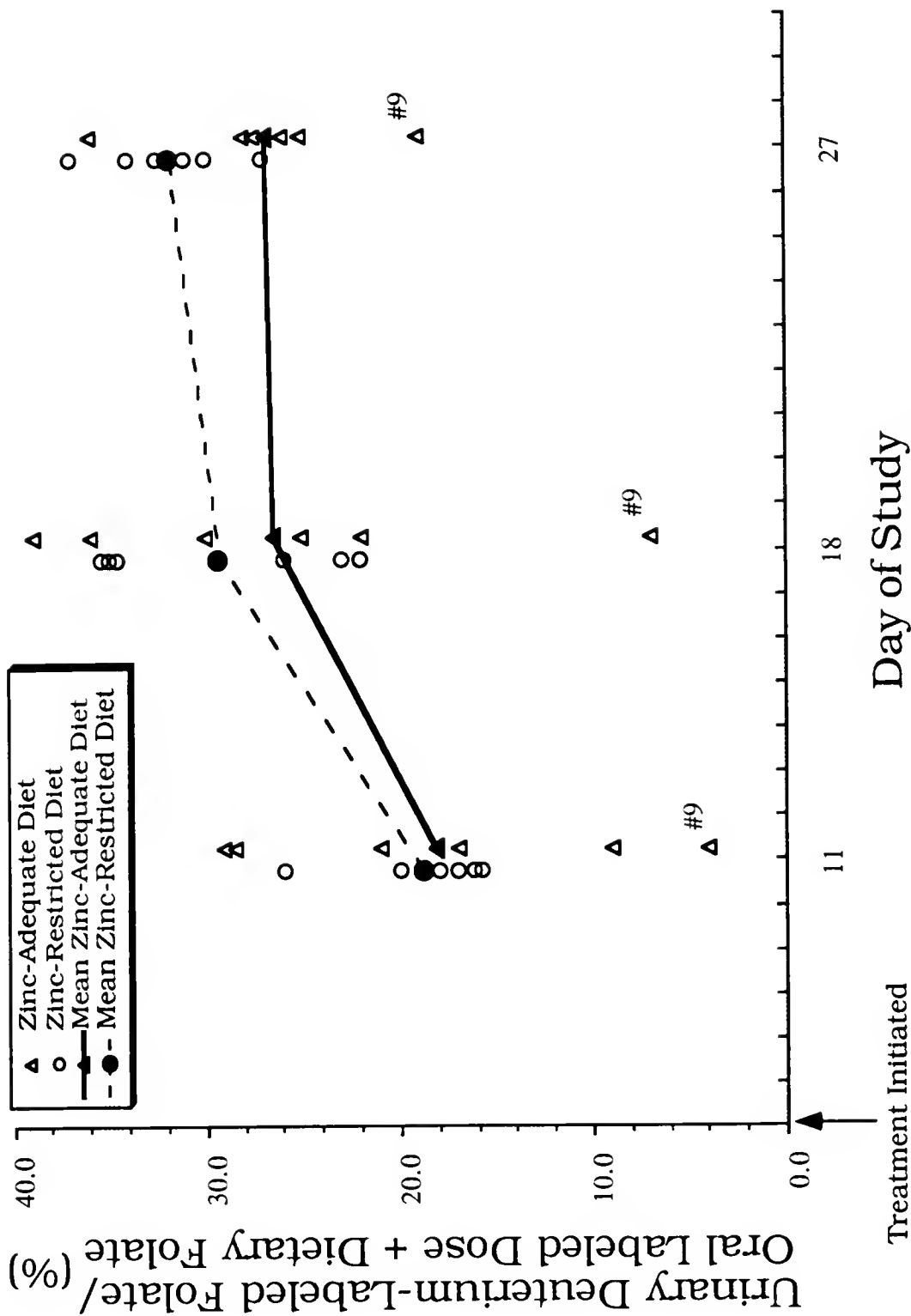


Figure 5-11. Percent of oral labeled folic acid plus dietary folate excreted as deuterium-labeled folate in subjects consuming zinc-restricted or zinc-adequate diets.



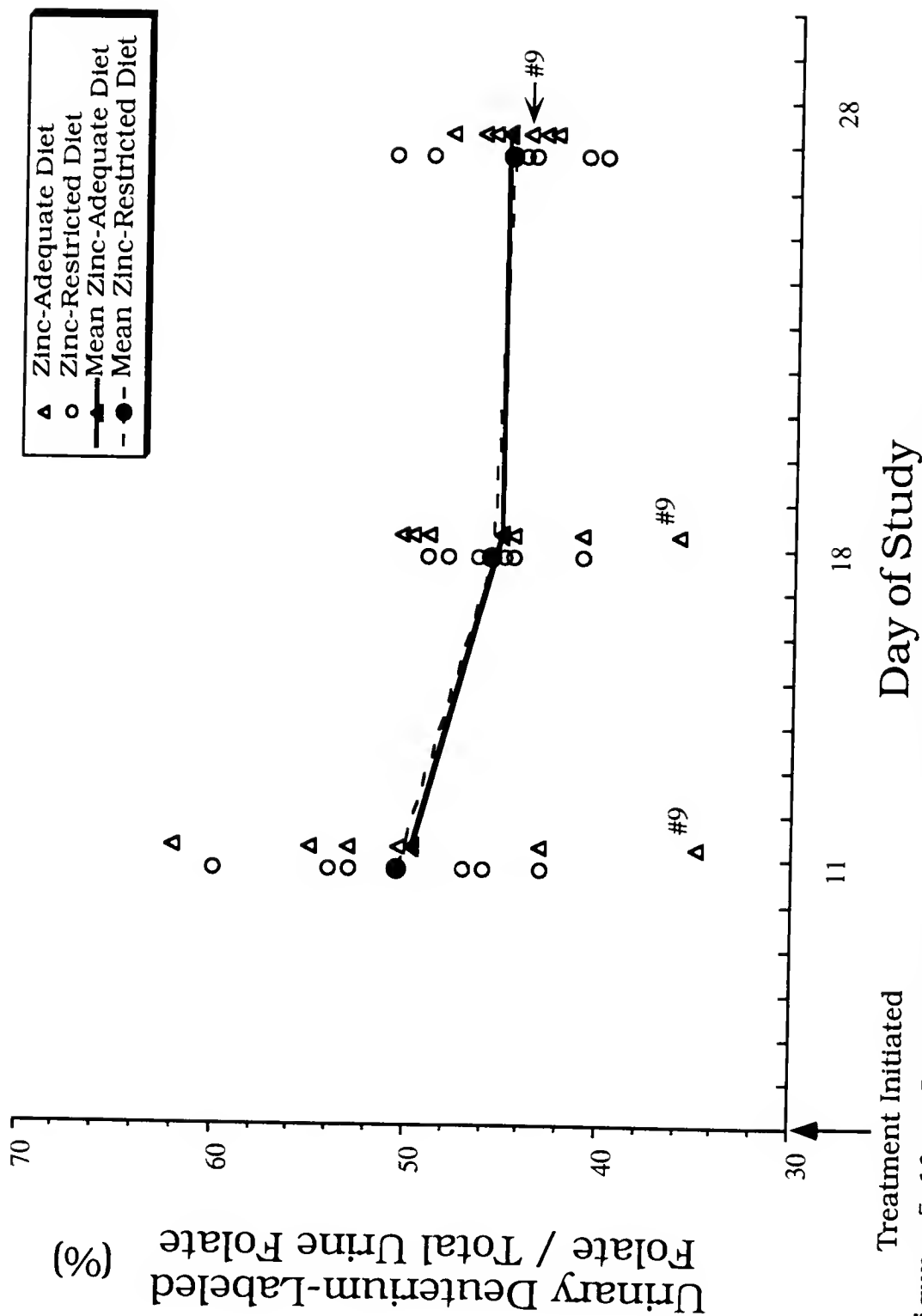


Figure 5-12. Percent of total urinary folate excreted as deuterium-labeled folate in subjects consuming zinc-restricted or zinc-adequate diets.

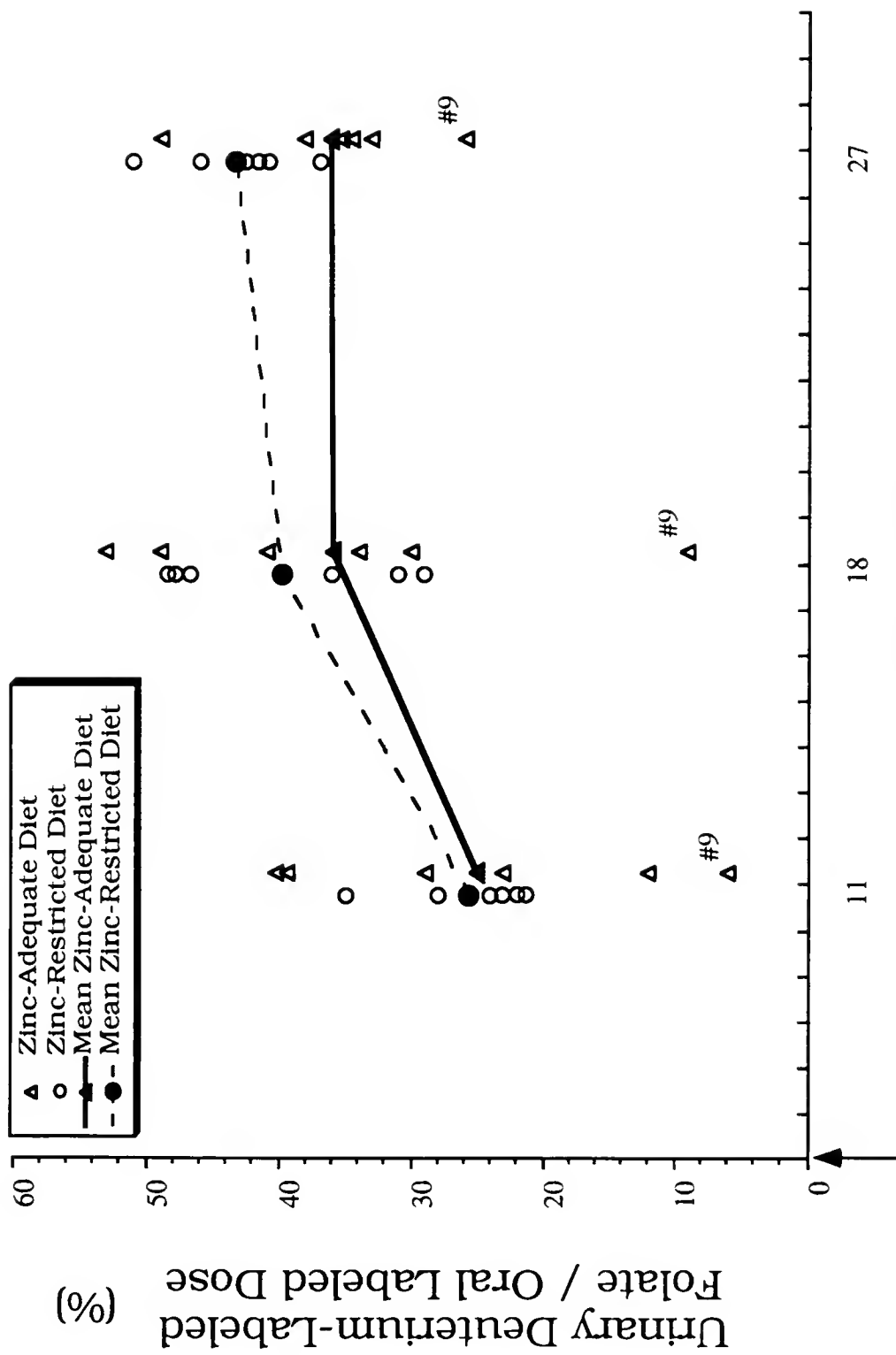


Figure 5-13. Percent of oral deuterium-labeled folic acid excreted as deuterium-labeled folate in subjects consuming zinc-restricted or zinc-adequate diets.

## CHAPTER 6 DISCUSSION

### Effect of Supplemental Folic Acid on Zinc Status

The present study used traditional indices of zinc status, and a newer index which has been shown to respond quickly to dietary zinc intake (Grider et al., 1990), to examine the effects of supplemental folic acid on zinc status in subjects consuming zinc-restricted or zinc-adequate diets. The lack of significant differences in both mean values and the change in mean values for these response variables suggests that short-term supplementation with 800  $\mu\text{g}$  folic acid/d does not adversely affect zinc status in healthy young men consuming zinc-restricted or zinc-adequate diets.

In the present study, significant changes in plasma zinc concentrations in response to the zinc-restricted diet without supplemental folic acid were not expected because of the relatively short duration of each study period (i.e. 25 days). It was reasoned, however, that if the purported effect of folic acid supplementation on zinc absorption was severe enough, a significant change in plasma zinc concentrations might be detected within this time interval when subjects consuming the zinc-restricted diet received the supplement. This type of response was not observed, which suggests that

supplemental folic acid does not affect plasma zinc concentrations or that the level and/or duration of supplementation was insufficient to elicit this change.

Despite the fact that the zinc-restricted diet was fed for a relatively short period of time, and folic acid supplementation did not appear to affect zinc status adversely during this time interval, it was surprising that subject #5 developed biochemical and behavioral symptoms of zinc deficiency while receiving the placebo. It is possible that consumption of the zinc-restricted diet for 25 days, combined with weight-lifting activity and a variable zinc intake during the washout phase (i.e. range of 8.9 to 19.7 mg zinc/d; mean of  $13.3 \pm 5.6$  mg/d), contributed to this subject's lowered zinc status during study period two. Alternatively, the provision of supplemental folic acid during study period one may have conferred a protective effect that resulted in the maintenance of plasma zinc concentrations during this phase of the study. Evidence against the latter suggestion was reported by Milne et al. (1990). These researchers found that zinc mobilization during exercise was impaired when folic acid supplements were fed.

Similar to plasma zinc, erythrocyte zinc and serum alkaline phosphatase concentrations are less sensitive and specific to changes in zinc status. Consequently, changes in these indices would not be expected in subjects consuming marginally zinc-deficient intakes for relatively short periods

of time unless the effect of the supplement was profound. Apparently, the effect of folic acid supplementation, if any, was not sufficient to produce significant changes in this study. A possible exception was subject #11, whose serum alkaline phosphatase difference value on day 29 was much more negative than that of the other subjects. In addition to being different from the response observed for any of the other subjects, this type of response was also different than the response observed when Milne et al. (1990) administered supplemental folic acid (i.e. 400 and 800  $\mu\text{g}/\text{d}$ ) to subjects consuming a zinc-adequate diet (i.e. 12.5 mg/d) for six weeks. These findings suggest that the decline in this subject's serum alkaline phosphatase activity may have been related to the fever he developed rather than ingestion of the supplement. Since infection and inflammation have been associated with changes in zinc mobilization and distribution (Beisel et al., 1974; Pekarek et al., 1978; Solomons et al., 1978), it is possible that zinc associated with serum alkaline phosphatase may have been mobilized and redistributed to be used for more critical functions.

By using a more sensitive and specific indicator of zinc status that responds quickly to changes in zinc intake (i.e. erythrocyte metallothionein; Grider et al., 1990; Thomas et al., 1992), it was predicted that the effect of supplemental folic acid on zinc status could be evaluated within the time frame of this study. The fact that significant differences in

mean erythrocyte metallothionein values or in the change in mean erythrocyte metallothionein values on versus off the supplement were not detected for either of the zinc diet groups, suggests that supplemental folic acid does not adversely affect zinc status. If folic acid supplementation had adversely affected zinc status, the concentration of erythrocyte metallothionein would have been significantly lower when the subjects received the supplement, particularly in subjects consuming the zinc-restricted diet.

Although the primary metallothionein isoform expressed in the bone marrow of rats is metallothionein-1 (Huber and Cousins, 1993), the various metallothionein isoforms and the relative proportions of these isoforms in human erythrocytes have not been determined. Since human metallothionein-1 (i.e. the form of the antibody used in this study) does not appear to cross-react with metallothionein-2, only the concentration of erythrocyte metallothionein-1 was determined in this study. Consequently, it is possible that changes in the concentration of metallothionein-2 due to the effect of the supplement and/or level of zinc intake could have occurred without being detected.

The reason for the significant difference between the patterns of change in erythrocyte metallothionein for the zinc-restricted and zinc-adequate diet groups is unknown. The fact that no significant time x supplement x diet interaction was detected for serum ferritin suggests that the changes

observed in erythrocyte metallothionein were specific and not due to an indirect effect on protein/iron metabolism. Since the erythrocyte is a cellular target of folate, it is possible that in response to continued low zinc intake, folate temporarily increased cellular zinc uptake through some unknown mechanism, and this increased concentration of intracellular zinc enhanced the induction and transcription of the metallothionein gene. Thus, by day 22, the zinc-restricted group appeared to have a notable positive response to the supplement. In support of this idea, mean plasma zinc difference values for the zinc-restricted group declined slightly from baseline through day 22, after which they began to increase. Alternatively, it is possible that the dissimilarities in the patterns of change between the zinc-restricted and zinc-adequate diet groups were due to the order in which the samples were analyzed. Conceivably, the variability in standard curves was large enough to produce erythrocyte metallothionein concentrations that were randomly lower or higher than their actual values depending on sampling order. This type of random error could have produced artificially lower or higher difference values that subsequently resulted in divergent patterns of change. Nevertheless, it is important to remember that within the time frame of this study, there was no evidence to suggest that erythrocyte metallothionein concentrations responded

differently to the effect of the supplement versus the placebo, regardless of the level of zinc intake.

Urinary zinc excretion has been shown to respond rapidly to changes in dietary zinc intake (Baer and King, 1984), but the usefulness of this response variable as an indicator of zinc status is limited because the amount of zinc excreted is highly variable (King, 1986) and can be altered by certain diseases and/or conditions (Prasad, 1983). To reduce the impact of these factors on the outcome and interpretation of urinary zinc data, this study used healthy subjects who served as their own control. As expected, subjects consuming the zinc-restricted diet, but not the zinc-adequate diet, responded to the reduction in zinc intake by excreting less zinc in their urine, regardless of the folic acid treatment. The fact that urinary zinc concentrations responded to a reduction in zinc intake, but not to the presence or absence of the supplement, provides further support for the conclusion that supplemental folic acid does not adversely affect zinc status under the conditions of this study.

As a caveat, it is possible that for some individuals, supplemental folic acid may affect tissue distribution and/or excretion of zinc. Supplemental folic acid may cause a reduction in urinary zinc excretion by: enhancing tissue uptake; enhancing endogenous zinc secretion and excretion; or, interfering with intestinal absorption, resulting in increased fecal excretion. These possibilities could explain why



subject #4 had a greater reduction in urinary zinc excretion when he was on the supplement. It is also conceivable that this subject had a large decrease in urinary zinc excretion due to greater dermal losses during the supplementation period (July/August) compared to the placebo period (October/November).

The only other controlled feeding studies that have examined the effect of supplemental folic acid on zinc status and absorption in human subjects were conducted by Milne et al. (1984; 1990). Based on differences in fecal and urinary zinc excretion (Milne et al., 1984; 1990), as well as absorption studies using labeled zinc isotopes (Milne, 1989; Milne et al., 1990), these researchers have concluded that supplemental folic acid adversely affects zinc absorption and may subsequently impair zinc status in individuals with low zinc intakes or increased zinc need.

In comparison to the present study, subjects in the study by Milne et al. (1984) experienced a significant decrease in mean plasma zinc concentrations when they consumed a diet containing 3.5 mg zinc/d for 16 weeks. The difference in plasma zinc concentrations between the folic acid-supplemented and placebo-treated groups was not significant. Apparently, the duration and extent of the reduction in zinc intake was sufficient to cause a notable reduction in mean plasma zinc concentrations in both groups, but further reductions due to the effect of the supplement were not observed. Although

plasma zinc is not a sensitive and specific indicator of marginal zinc status (King, 1990), the longer duration of these studies and the fact that plasma zinc concentrations responded to a reduction in zinc intake but not to folic acid supplementation seem to support the view that supplemental folic acid does not adversely affect zinc status, even when zinc intake/need is low. In a more recent study, Milne et al. (1990) did not find significant differences in plasma zinc concentrations when subjects consuming zinc-adequate diets received two six-week periods of folic acid supplementation alternated with two six-week periods without supplementation.

One might argue that the lack of a significant difference in mean plasma zinc concentrations between the folic acid-supplemented and placebo-treated groups in the study by Milne et al. (1984) was due to greater homeostatic adaptation in the former group. Urinary zinc data obtained during the zinc-depletion stage of their study support this idea. However, the fact that supplemented subjects also had significantly lower mean urinary zinc concentrations after consuming a diet containing 33.5 mg zinc/d for four weeks raises the question of whether the response was due to the supplement or to some other difference(s) in the subjects assigned to each of the treatment groups. It seems unlikely that such a small amount of folic acid, relative to the level of zinc intake (i.e. zinc:folic acid ratio = 647:1), could interfere with zinc absorption. In fact, at this level of zinc intake no

significant difference in fecal zinc excretion between subjects in the folic acid-supplemented versus the placebo-treated groups was detected, suggesting that zinc absorption was not adversely affected by supplemental folic acid. Given the small number of subjects in this study (n=8) it would have been more informative if each subject had served as his own control on and off the supplement. Significant differences in absorption and excretion patterns due to the effect of supplemental folic acid were not detected in subjects consuming a zinc-adequate diet (Milne et al., 1990).

#### Effect of Level of Zinc Intake on Folate Utilization

Urinary excretion of deuterium-labeled and unlabeled folate, as well as serum and erythrocyte folate data, support the conclusion that under the conditions of this study, the bioavailability of supplemental folic acid monoglutamate was not influenced by the level of zinc intake. The findings of Tamura et al. (1978) were similar except that they used severely zinc-deficient subjects and determined the response to folic acid supplementation by measuring the rise in serum folate concentrations after administering a single oral dose of folic acid monoglutamate.

Although the results of the present study suggest that the level of zinc intake did not adversely affect the utilization of supplemental folic acid monoglutamate, it is possible that the amount of folate in the diet (i.e. 290 µg/d)

and supplement (i.e. 800  $\mu\text{g/d}$ ) was sufficient to overcome subtle changes in folate metabolism that may have occurred secondary to a marginal zinc intake. Thus, it is possible that a marginal zinc intake may adversely affect folate metabolism when smaller doses of folic acid monoglutamate are consumed.

In contrast to the effect of zinc intake/status on absorption of folic acid monoglutamate, Tamura et al. (1978) found that absorption of folic acid polyglutamate was compromised in severely zinc-deficient subjects. Whether or not this is a problem in individuals with marginal zinc intakes remains to be determined. It is possible that the defect in hydrolysis and/or absorption of pteroyl-polyglutamates only occurs in severely zinc-deficient individuals, or impaired absorption may only become evident when large amounts of pteroylpolyglutamates are consumed. Alternatively, the reduction in cellular proliferation and growth associated with mild zinc deficiency may decrease the metabolic demand for folate sufficiently to offset any impairment in absorption of this nutrient.

The presence of deuterium-labeled folate in the subjects' urine showed that the folic acid supplement was absorbed across the intestinal mucosa, transported in the plasma and excreted by the kidneys. The lack of significant differences in urinary excretion of labeled folate metabolites and serum and erythrocyte folate concentrations in subjects consuming

zinc-restricted versus zinc-adequate diets suggests that overall tissue uptake and utilization of folate was similar between the groups. However, whether or not there were differences between tissues in folate uptake and metabolism in subjects consuming zinc-restricted versus zinc-adequate diets, as suggested by previous animal studies (Tamura et al., 1987; Williams and Mills, 1973), is unknown. As noted earlier, one of the subjects consuming the zinc-adequate diet (i.e. subject #9) had lower urinary folate concentrations compared to subjects in either of the zinc diet groups. A possible explanation for this finding is that this subject absorbed folic acid less efficiently. However, the fact that his serum folate concentration more than doubled during supplementation (i.e. baseline: 7.5 ng/mL; final 18.7 ng/mL) argues against this idea. Interestingly, the increase in his erythrocyte folate concentration during supplementation was modest compared to the average increase for the zinc-adequate group. This raises the possibility that folate was being directed to some other tissue(s). It is also possible that this subject catabolized folate more extensively, and the products of folate catabolism were not quantified, resulting in under-estimation of urinary folate excretion.

The total folate intake of subjects in this study consisted of 74% deuterium-labeled folic acid and 26% unlabeled dietary folate. Based on these percentages, the isotopic enrichment of tissue and excreted folates at complete

equilibrium should be approximately 74%. By day 29, only 45% of the folate excreted in the urine was deuterium-labeled, which suggests that equilibrium had not been achieved within the time frame of the study. Research currently being conducted suggests that a long period of time is required to reach equilibrium.

## CHAPTER 7

### SUMMARY AND CONCLUSIONS

The concept that zinc and folic acid metabolism may be related was first suggested in the early 1970's when Williams and Mills (1973) observed a reduction in the mean hepatic folate concentration in rats fed zinc-deficient diets. The discovery that bovine hepatic folate conjugase was a zinc-dependent enzyme (Silink et al., 1975) prompted further research regarding the potential effect of zinc deficiency on folate metabolism, and in 1978, Tamura et al. reported that absorption of folate polyglutamate, but not folate monoglutamate, was adversely affected in zinc-deficient males. In addition to the effect of zinc deficiency on folate absorption, a subsequent animal study (Tamura et al., 1987) suggested that impaired zinc status may alter folate metabolism.

The hypothesis that folic acid may adversely affect zinc absorption and homeostasis was first proposed by Milne and colleagues in 1984. Milne (1989) subsequently revised this hypothesis, suggesting that impaired zinc absorption/status may only be manifested in conditions of elevated zinc need or low zinc intake. Although numerous animal and human studies have examined the effect of supplemental folic acid on zinc

absorption/status, the results of these investigations have been equivocal. The lack of agreement among these studies may be due in part to differences in study designs, the lack of a sensitive and reliable index to assess zinc status and/or failure to control dietary intake.

Determination of the relative safety of supplemental doses of folic acid has important public health implications since folic acid supplements are commonly recommended for individuals with chronic diseases and disorders, pregnant women and individuals with a documented folate deficiency. Supplemental doses of this nutrient have also recently been recommended for women of childbearing age as a means for reducing the occurrence/recurrence of neural tube defects. Since folate and zinc are important in protein and nucleic acid metabolism and genetic expression, changes in the bioavailability and/or metabolism of either or both of these nutrients can have deleterious effects.

The objectives of the present study were to determine if supplemental folic acid affects zinc status in subjects consuming zinc-restricted or zinc-adequate diets and if folate utilization was affected by the level of zinc intake. Subjects were fed a constant diet containing either 3.5 or 14.5 mg zinc/d for two 25-day periods, and deuterium-labeled folic acid (i.e. 800  $\mu$ g/d) was consumed during one of the study periods. The effect of supplemental folic acid on zinc status was determined using traditional measures of zinc



status (i.e. plasma, erythrocyte and urinary zinc concentrations; serum alkaline phosphatase activity), as well as a newer method (i.e. erythrocyte metallothionein) that responds quickly to changes in zinc intake (Grider et al., 1990). The response of serum, erythrocyte and urinary folate (i.e. total folate and labeled folate) concentrations to folic acid supplementation were used to determine the effect of level of zinc intake on folate utilization.

The present study did not detect a difference in zinc status as a result of short-term supplementation with 800  $\mu$ g of folic acid/d in subjects consuming zinc-restricted or zinc-adequate diets, nor did it find a difference in folate utilization due to the level of zinc intake. The safety and effectiveness of supplementation in other population subgroups (i.e. women, pregnant women, individuals taking anticonvulsant medications, population groups at risk for developing vitamin B<sub>12</sub> deficiency, etc.) remains to be determined. Thus, further research in this area seems warranted since folic acid supplements are already being used by certain segments of the population, and if the Food and Drug Administration - Food Advisory Council's (Anon., *Food Chemical News*, 1993) recommendation to enrich flour with folic acid is accepted, the population as a whole will be consuming additional amounts of this nutrient.

This study represents the first diet-controlled human study to be conducted since Milne (1989) first suggested that

supplemental folic acid may adversely affect zinc status in subjects with low zinc intakes or increased zinc needs. Although additional well-controlled and designed studies are needed in order to bring closure to this issue, this study serves as a starting point toward this goal.

APPENDIX A  
SUBJECT SELECTION SCREENING TOOL

Food Science and Human Nutrition  
University of Florida  
359 Food Science Building  
Gainesville, FL 32611  
904-392-1991

Demographic Information

Date \_\_\_\_\_  
Mo. Day Yr.

Name \_\_\_\_\_  
Last First Middle

SS# \_\_\_\_\_ Phone \_\_\_\_\_  
Day Evening

Age \_\_\_\_\_ Date of Birth \_\_\_\_\_  
Mo. Day Yr.

ADDRESS \_\_\_\_\_

Sex \_\_\_\_\_ Male \_\_\_\_\_ Female

Race \_\_\_\_\_ Caucasian \_\_\_\_\_ Hispanic

\_\_\_\_\_ Black \_\_\_\_\_ Other

\_\_\_\_\_ Asian

Religion (optional) \_\_\_\_\_ Catholic \_\_\_\_\_ Hindu

\_\_\_\_\_ Protestant \_\_\_\_\_ Muslim

\_\_\_\_\_ Jewish \_\_\_\_\_ None

\_\_\_\_\_ Jehovah Witness

\_\_\_\_\_ Other \_\_\_\_\_

## Education Completed

☐ High School Graduate☐ Bachelor's Degree☐ Master's Degree☐ Doctoral Degree

## Present Work/Student Status (Check all that apply):

☐ Working Full-time ☐ Full-time Student☐ Working Part-time ☐ Part-time Student☐ Not EmployedMedical History

Indicate if you have had or currently have any of the following medical problems (Check all that apply):

Past	Now	
<input type="checkbox"/>	<input type="checkbox"/>	Alcoholism
<input type="checkbox"/>	<input type="checkbox"/>	Allergies
<input type="checkbox"/>	<input type="checkbox"/>	Anemia
<input type="checkbox"/>	<input type="checkbox"/>	Arthritis
<input type="checkbox"/>	<input type="checkbox"/>	Asthma
<input type="checkbox"/>	<input type="checkbox"/>	Blood Clots
<input type="checkbox"/>	<input type="checkbox"/>	Bronchitis
<input type="checkbox"/>	<input type="checkbox"/>	Cardiovascular Disease (Atherosclerosis/Heart Attack)
<input type="checkbox"/>	<input type="checkbox"/>	Claudication
<input type="checkbox"/>	<input type="checkbox"/>	Cystic Fibrosis
<input type="checkbox"/>	<input type="checkbox"/>	Dermatitis
<input type="checkbox"/>	<input type="checkbox"/>	Emotional Disorder
<input type="checkbox"/>	<input type="checkbox"/>	Eye Problems

Past	Now
_____	_____ Gall Bladder Disease
_____	_____ Glaucoma
_____	_____ Gout
_____	_____ Hair Loss (excessive)
_____	_____ Headaches
_____	_____ Hemorrhoids
_____	_____ Hernia
_____	_____ Hypercholesterolemia/Hyperlipidemia
_____	_____ Hypertension
_____	_____ Intestinal Disorders
_____	_____ Kidney Disease
_____	_____ Liver Disease
_____	_____ Lung Disease
_____	_____ Mental Illness
_____	_____ Neurologic Disorder
_____	_____ Obesity/Overweight
_____	_____ Prostate Trouble
_____	_____ Rheumatic Fever
_____	_____ Seizure Disorder
_____	_____ Stomach Disease
_____	_____ Stroke
_____	_____ Thyroid Disease
_____	_____ Tumors/Cancer - List Type: _____
_____	_____ Ulcers
_____	_____ Other - Specify: _____

Indicate if you have had any of the following surgeries, and if so, the approximate date (Check all that apply):

	Month/Year
Cardiovascular Surgery	_____
Gastric Surgery	_____
Gall Bladder Surgery	_____
Intestinal Surgery	_____
Kidney Surgery	_____
Lung Surgery	_____
Pancreatic Surgery	_____
Prostate Surgery	_____
Thyroid Surgery	_____
Other - Specify: _____	

Indicate the prescription/nonprescription medicines you currently use on a regular basis (Check all that apply):

☐ Allergy Medicines/Antihistamines  
☐ Antacids  
☐ Antibiotics  
☐ Anti-arrhythmics  
☐ Anti-inflammatory Agents (i.e. ibuprofen)  
☐ Aspirin  
☐ Asthma Medicines  
☐ Beta Blockers  
☐ Blood Pressure Medicines  
☐ Blood Thinners (i.e. anticoagulants)  
☐ Cortisone  
☐ Decongestants

- ☐ Diabetes Medicines/Insulin  
☐ Diuretics  
☐ Gout Medicines  
☐ Heart Medicines  
☐ Hormones  
☐ Laxatives  
☐ Nitroglycerin  
☐ Pain Medicines  
☐ Psychiatric Medicines/Anti-depressants  
☐ Sedatives/Sleeping Pills  
☐ Seizure Medicines  
☐ Thyroid Medicines  
☐ Tranquilizers  
☐ Other - Specify: \_\_\_\_\_

Do you take any type of nutritional supplement (i.e. vitamin and/or mineral pill; protein supplement; rose hips; other supplements; etc.)?

\_\_\_\_\_ Yes \_\_\_\_\_ No

If you answered "yes" to the last question, indicate the brand(s), type of supplement(s), frequency and amount(s).

If you currently use supplements, would you be willing to discontinue use of your usual supplements for 5 to 6 months?

\_\_\_\_\_ Yes \_\_\_\_\_ No

Do you use any of the following tobacco products (check all that apply):

- |                                     |  |
|-------------------------------------|--|
| <input type="checkbox"/> Cigarettes | <input type="checkbox"/> Chewing Tobacco |
| <input type="checkbox"/> Cigars     | <input type="checkbox"/> Snuff           |
| <input type="checkbox"/> Pipe       |  |

Activity Status

Indicate your usual activities, frequency per month and minutes per session by placing a check mark in the appropriate columns (check all that apply):

	<u>Frequency/Month</u>				<u>Minutes/Session</u>		
	1-4	5-8	9-12	13-16	0-20	20-40	40-60
___ Badminton	___	___	___	___	___	___	___
___ Baseball/ Softball	___	___	___	___	___	___	___
___ Basketball	___	___	___	___	___	___	___
___ Boating	___	___	___	___	___	___	___
___ Bowling	___	___	___	___	___	___	___
___ Cycling(motor)	___	___	___	___	___	___	___
___ Cycling (road)	___	___	___	___	___	___	___
___ Cycling (stationery)	___	___	___	___	___	___	___
___ Dancing (aerobic)	___	___	___	___	___	___	___
___ Dancing (social)	___	___	___	___	___	___	___
___ Fishing	___	___	___	___	___	___	___
___ Golf (ride)	___	___	___	___	___	___	___
___ Golf (walk)	___	___	___	___	___	___	___
___ Gymnastics	___	___	___	___	___	___	___
___ Hiking	___	___	___	___	___	___	___
___ Horseback Riding	___	___	___	___	___	___	___
___ Hunting	___	___	___	___	___	___	___
___ Jogging/ Running	___	___	___	___	___	___	___



[illegible]

Does your usual job/schoolwork require sustained physical activity?

\_\_\_\_\_ Yes \_\_\_\_\_ No

How would you rate your physical fitness/endurance (check one)?

\_\_\_\_\_ Low \_\_\_\_\_ Medium \_\_\_\_\_ High

How would you rate your strength (check one)?

\_\_\_\_\_ Low \_\_\_\_\_ Medium \_\_\_\_\_ High

### Diet History

Height (without shoes) \_\_\_\_\_ inches

Usual weight (dressed without shoes) \_\_\_\_\_ lbs

Current weight (dressed without shoes) \_\_\_\_\_ lbs

Is your weight fairly stable? \_\_\_\_\_ Yes \_\_\_\_\_ No

Are you satisfied with your current weight? \_\_\_\_\_ Yes \_\_\_\_\_ No

If not, please explain: \_\_\_\_\_

Do you weigh yourself frequently? \_\_\_\_\_ Yes \_\_\_\_\_ No

Have you gained or lost weight in the past year?

\_\_\_\_\_ Yes \_\_\_\_\_ No

If yes, how much did you gain/lose? \_\_\_\_\_ lbs

Was this weight change intentional? \_\_\_\_\_ Yes \_\_\_\_\_ No

How many times (meals and snacks) do you eat each day? \_\_\_\_\_

Are you allergic to any foods? \_\_\_\_\_ Yes \_\_\_\_\_ No

If yes, please list all foods: \_\_\_\_\_

Are there any foods you cannot or will not eat?

\_\_\_\_\_ Yes \_\_\_\_\_ No

If yes, please list these foods: \_\_\_\_\_

Which of the following words best describes your appetite (check only one)?

\_\_\_\_\_ Small  
 \_\_\_\_\_ Medium  
 \_\_\_\_\_ Large

Do you follow any of the modified diets listed below (check all that apply)?

_____ Diabetic	_____ Weight Reduction
_____ Low Sodium	_____ Weight Gain
_____ Renal	_____ Gastric Banding
_____ Ulcer/Bland	_____ Kosher
_____ Vegetarian	_____ Other: _____
_____ Low Cholesterol/Low Fat	

Do you drink coffee or tea? \_\_\_\_\_ Yes \_\_\_\_\_ No

If yes, could you limit your intake or these to one cup per day for a month without difficulty?

\_\_\_\_\_ Yes \_\_\_\_\_ No

Do you consume alcoholic beverages (i.e. beer, wine, wine coolers, hard liquor, etc.)

\_\_\_\_\_ Yes \_\_\_\_\_ No

If yes, could you discontinue your intake of these for a month?

\_\_\_\_\_ Yes \_\_\_\_\_ No

Indicate the amount and frequency with which you consume the following:

	<u>Amt.</u>	<u>Never/ Rarely</u>	<u>Occas.</u>	<u>Freq.</u>	<u>Always</u>
Beef	_____	_____	_____	_____	_____
Beer (regular)	_____	_____	_____	_____	_____
Beer (light)	_____	_____	_____	_____	_____
Bread	_____	_____	_____	_____	_____
Cake	_____	_____	_____	_____	_____

	<u>Amt.</u>	<u>Never/ Rarely</u>	<u>Occas.</u>	<u>Freq.</u>	<u>Always</u>
Candy	_____	_____	_____	_____	_____
Cereal	_____	_____	_____	_____	_____
Chicken	_____	_____	_____	_____	_____
Coffee (regular)	_____	_____	_____	_____	_____
Coffee (decaf)	_____	_____	_____	_____	_____
Cola (regular)	_____	_____	_____	_____	_____
Cola (diet)	_____	_____	_____	_____	_____
Cookies	_____	_____	_____	_____	_____
Eggs	_____	_____	_____	_____	_____
Fish (& tuna)	_____	_____	_____	_____	_____
Fruit	_____	_____	_____	_____	_____
Fruit Juice	_____	_____	_____	_____	_____
Hard Liquor	_____	_____	_____	_____	_____
Legumes	_____	_____	_____	_____	_____
Milk	_____	_____	_____	_____	_____
Pork	_____	_____	_____	_____	_____
Shellfish	_____	_____	_____	_____	_____
Snack Foods	_____	_____	_____	_____	_____
Starches	_____	_____	_____	_____	_____
Tea	_____	_____	_____	_____	_____
Turkey	_____	_____	_____	_____	_____
Vegetables	_____	_____	_____	_____	_____
Wine	_____	_____	_____	_____	_____
Wine Coolers	_____	_____	_____	_____	_____

Indicate if you disagree (DA), moderately agree (MA) or (SA) strongly agree with each of the following statements by placing a check in the appropriate column.

	DA	MA	SA
Food is very important to me.	_____	_____	_____
Eating is one of my favorite pastimes.	_____	_____	_____
I consider myself to have a lot of will power.	_____	_____	_____
I don't usually pay much attention to what I eat.	_____	_____	_____

APPENDIX B  
ZINC RESTRICTED METABOLIC DIET

Day 1

<u>Breakfast</u>	<u>Amount</u>
Corn flakes	38 g
White bread	50 g
Margarine	10 g
Grape jelly	24 g
Coffee Rich®	120 g
Apple juice	120 g
Apple juice, special	45 g
Protein shake, orange	360 g
Morning supplements	1 vitamin B complex 1 calcium citrate 1 magnesium gluconate

Lunch

Grape juice	236 g
Turkey breast	30 g
White bread	50 g
Mayonnaise	12 g
Potato chips	30 g
Vanilla wafers	16 g
Pineapple chunks	100 g

Dinner

Apple juice	120 g
Chicken, boneless breast	60 g
Barbecue sauce	16 g
Margarine	15 g
Anellini®	120 g
Green beans	100 g
White bread	12 g
Salt, iodized	260 mg
Cupcake, white (prebaked)	40 g
Blueberries	30 g
Cool Whip®	5 g
Protein shake, tropical	360 g
Evening supplements	1 biotin 2 magnesium gluconate

Snack

Twinkie®	43 g
Cranberry juice	120 g

Day 2BreakfastAmount

Trix®	21 g
White bread	50 g
Margarine	10 g
Apple jelly	24 g
Coffee Rich®	120 g

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Apple juice	120 g
Apple juice, special	45 g
Protein shake, lemon	360 g
Morning supplements	1 vitamin B complex 1 calcium citrate 1 magnesium gluconate

#### Lunch

Orange juice	236 g
Turkey breast	30 g
White bread	50 g
Mayonnaise	12 g
Fritos® corn chips	50 g
Lorna Doones®	40 g
Applesauce	120 g

#### Dinner

Chicken, boneless breast	60 g
Corn flakes	5 g
Margarine	22.7 g
Mashed potatoes, dry mix	19 g
Coffee Rich®	20 g
Salt, iodized	700 mg
Rusks	10 g
Carrots, canned	100 g
White bread	12 g
Strawberry banana gelatin	120 g
Protein shake, orange	360 g



## Evening supplements

1 biotin  
2 magnesium gluconate

Snack

Angel food cake	30 g
Grape juice	118 g

Day 3BreakfastAmount

Corn flakes	38 g
White bread	50 g
Margarine	10 g
Grape jelly	24 g
Coffee Rich®	120 g
Apple juice	120 g
Apple juice, special	45 g
Protein shake, orange	360 g
Morning supplements	1 vitamin B complex 1 calcium citrate 1 magnesium gluconate

Lunch

Cranberry juice	236 g
Turkey breast	30 g
White bread	50 g
Mayonnaise	12 g
Fritos® corn chips	50 g
Pound cake	25 g
Pears	100 g

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Dinner

Chicken, boneless breast	60 g
White rice, dry	30 g
Salt, iodized	260 mg
Lettuce	28 g
French dressing	12 g
Rusks®	10 g
White bread	12 g
Margarine	15 g
Applesauce	100 g
Cinnamon crumb topping	20 g
Protein shake, tropical	360 g
Evening supplements	1 biotin 2 magnesium gluconate

Snack

Sugar cookie	15 g
Orange juice	118 g

APPENDIX C  
CONTRACT

I, \_\_\_\_\_, agree to comply with the following rules as part of my participation in the study being conducted to investigate the interrelationships between zinc and folic acid:

1. Report on time for meals and blood draws.
2. Eat all foods and beverages served to me by the researcher, including vitamin and mineral supplements.
3. Avoid eating/drinking anything except the foods and beverages provided to me by the researcher. (Remember NO ALCOHOL.)
4. Eat my meals and snack using metabolic style eating techniques, which requires scraping, rinsing and licking clean all food containers and utensils.
5. Avoid the use of prescription and over the counter medications. If the use of medication is essential, I will consult the researcher first, if possible. If the use of prescription medication is unavoidable, I understand that I may need to withdraw from the study.
6. Collect 24 hour urine specimens in the containers provided; keep specimens refrigerated; and return all containers on the appropriate dates.
7. Limit exercise to a MAXIMUM of one hour per day. (This is not the same as an average of one hour per day.)
8. Use only approved personal hygiene products.
9. Complete the "Morning Checklist" on a daily basis.
10. Wash hands thoroughly before eating.
11. Return empty water bottles to be sanitized and refilled.
12. Report any unusual symptoms.

13. Report any food losses or spills.
14. Complete food records, when requested, during study periods one and two.
15. Comply with weight check schedule.
16. Avoid the use of tobacco products such as cigarettes, cigars, pipes, chewing tobacco and snuff.

In return for my complete cooperation and compliance with the above, I understand that I will be provided with all of my meals for a total of 56 days. I will also receive financial compensation in the amount of \$800.00. I recognize that if I fail to comply with the above, I will be dropped from the study and will only receive compensation for the time that I actually participated in the study.

Signed this second day of July, 1990: \_\_\_\_\_

Witnesses: \_\_\_\_\_

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## BIOGRAPHICAL SKETCH

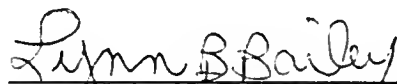
Gail Patricia Abbott Kauwell was born in New York, NY, on August 30, 1952. She is the eldest of Robert and Audrey Abbott's six children. Gail graduated from East Brunswick High School, East Brunswick, NJ, in 1970. She attended the University of Maine, Orono, and graduated with highest distinction in 1974 and 1975, earning a B.A. in psychology and a B.S. in nutrition, respectively. As an undergraduate, she was inducted into the honor societies of Phi Kappa Phi, Phi Beta Kappa and Omicron Nu. She completed a dietetic internship at Perth Amboy Medical Center in 1976 and became a registered dietitian that same year. She was employed as a registered dietitian for two years and then returned to school after being awarded a scholarship from the Bureau of Health Manpower. In 1979, she graduated from the University of Florida with a master's degree in nutrition. Upon graduation, she worked as a registered dietitian for several more years and then accepted an academic appointment at the University of Florida in the College of Health Related Professions' Program in Clinical and Community Dietetics.

During her employment with the University, Gail has been promoted to the rank of Assistant Professor, has received the College of Health Related Professions' Teacher of the Year

Award and the Faculty Research Award, was awarded tenure in 1987 and has served as Interim Director for the Program in Clinical and Community Dietetics (1983-1985; 1991-1992). In 1986, she received permission from the Graduate School of the University of Florida to pursue a doctoral degree in the Department of Food Science and Human Nutrition. She began her studies on a part-time basis and met her residency requirement while on sabbatical/leave of absence without pay from the University. During this time she was awarded a fellowship from the Center for Nutritional Sciences. She was also the recipient of scholarship awards from the Florida Dietetic Association and the American Dietetic Association and was inducted into the honor society of Gamma Sigma Delta in 1988. Her research was funded in part by competitive grants that she was awarded through the University of Florida's Division of Sponsored Research.

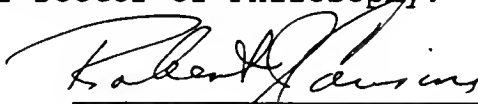
Gail has been the recipient of many other awards and honors, including Florida's Distinguished Dietitian, the Florida Dietetic Association President's Award and the American Dietetic Association Outstanding Service Award. She is listed in several biographical texts including Who's Who in the South and Southwest. She has been active in several professional associations and has served as president of the Florida Dietetic Association. After completion of her doctorate, Gail will continue as a faculty member at the University of Florida.

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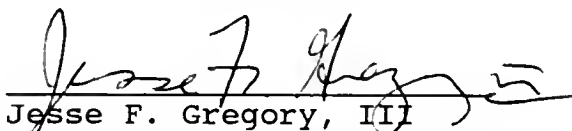
Lynn B. Bailey, Chair  
Professor of Food Science  
and Human Nutrition

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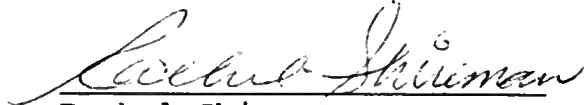
Robert J. Cousins  
Boston Family Professor of  
Human Nutrition

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Jesse F. Gregory, III  
Professor of Food Science  
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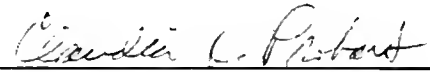
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Rachel Shiremen  
Professor of Food Science  
and Human Nutrition



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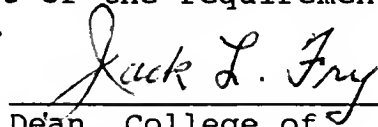


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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1993



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Dean, College of  
Agriculture

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Dean, Graduate School

